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DEVELOPMENT AND CHARACTERIZATION OF A MURINE MONOCLONAL ANTIBODY (MoAb), 6D4, AGAINST THE PRECURSOR OF PHEOMELANIN, 5-S-CYSTEINYLDOPA (5-S-CD)

BY

JUN LIU

©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE.**

IN

EXPERIMENTAL MEDICINE

DEPARTMENT OF **MEDICINE**

EDMONTON, ALBERTA

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Dr R.L. Jones (Chairman)

Dated: Dec 15 1992

Dedicated
to my husband and my parents

ABSTRACT

There are two major types of melanin pigment in humans, pheomelanin (reddish-yellow pigment) and eumelanin (brown-black). The initial step in the synthesis of the both melanins uses a common metabolic pathway, in which the key oxidative enzyme is tyrosinase. Tyrosine is oxidized in a two-step reaction, consisting in the conversion of tyrosine to dopa and thence to dopaquinone which, in turn, gives rise to eumelanin via a series of auto-oxidation reactions with post-tyrosinase regulatory factors. By contrast, in pheomelanin synthesis the dopaquinone reacts with cysteine or other sulfhydryl compounds [*e.g.*, glutathione (GSH)] to form cysteinyldopas (CDs), the major precursors of pheomelanin. Although a good deal is now known about the synthesis of eumelanin, little is known concerning the pathway and regulatory factors in the synthesis of pheomelanin, a melanin pigment that was described only fairly recently.

This thesis describes the development and characterization of a murine monoclonal antibody (MoAb) directed against 5-S-cysteinyldopa (5-S-CD), a major precursor of pheomelanin. The main reason for developing this MoAb was for studying the biologic role of 5-S-CD and the pathway of its biosynthesis in the production of pheomelanin, in the belief that this new antibody might have potential clinical use in the diagnosis of pigmentary disorders and neoplasms.

In the studies reported here, a competitive enzyme-linked immunosorbent assay (ELISA) was developed and it was demonstrated that the MoAb, designated as 6D4, recognizes both 5-S-CD and pheomelanin but has minimal cross-reactivity with eumelanin and its

metabolites. The 50% inhibition values for dopa and phenylalanine were 180 ng/mL and 2500 ng/mL, respectively. Thus, the antigenic epitope recognized by this MoAb is more closely related to the chemical structure of pheomelanin and its precursor 5-S-CD than to eumelanin. Immunohistochemical assays indicated that the antigenic epitope of this MoAb is detectable in certain types of melanocytic tumors; for example, three of eight samples of superficial spreading melanoma reacted positively, but samples of normal melanocytes in the skin and of non-melanocytic tumors and other normal tissues and organs did not react.

It was concluded that this MoAb may be useful for investigating the distribution of pheomelanin and its intermediates in melanocytic tumors and for detecting metabolites of pheomelanin in the body fluids of patients in whom these tumors are disseminated.

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LIST OF ABBREVIATIONS

2-ME	2-mercaptoethanol
5-S-CD	5-S-cysteinyldopa
5H6MI2C	5-hydroxy-6-methoxyindole-2-carboxylic acid
6H5MI2C	6-hydroxy-5-methoxyindole-2-carboxylic acid
ABC	avidin–biotin immunoperoxidase complex
ABTS	2,2'-azino-*bis* (3-ethylbenz-thiazoline-6-sulfonic acid)
APC	antigen–presenting cell
BSA	bovine serum albumin
CD	cysteinyldopa
DAB	diaminobenzidine
DHI	dihydroxyindole
DHICA	5,6-dihydroxyindole-2-carboxylic acid
DMN	dysplastic melanocytic nevi
DMSO	dimethyl sulfoxide
DOPA	3,4-dihydroxyphenylalanine
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
ELISA	enzyme-linked immunosorbent assay
EMU	epidermal melanin unit
ER	endoplasmic reticulum
FCS	fetal-calf serum
GERL	Golgi–endoplasmic reticulum–lysosome
GSH	glutathione
©GTP	©-glutamic-pyruvic transaminase
HAT	hypoxanthine, aminopterin, and thymidine
HMSA	human melanosome-specific antigen
HPLC	high-performance liquid chromotography
HPRT	hypoxanthine guanine phosphoribosyl transferase
kDa	kilodalton
KLH	keyhole limpet hemocyanin
LM	lentigo maligna
LMM	lentigo maligna melanoma
MAG	melanoma-associated glycoprotein

MHC	major histocompatibility complex
MoAb	monoclonal antibody
MSH	melanocyte-stimulating hormone
OD	optical density
PAA	pigmentation-associated antigen
PBS	phosphate-buffered saline
PEG	polyethylene glycol
RER	rough endoplasmic reticulum
RIA	radioimmunoassay
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SER	smooth endoplasmic reticulum
SSM	superficial spreading melanoma
TCA	trichloroacetic acid
TRP	tyrosinase-related protein
UVL	ultraviolet light

CHAPTER 1
LITERATURE REVIEW

A. COMPOSITION AND FUNCTIONS OF THE EPIDERMIS

The skin consists of three layers of tissue, *i.e.*, epidermis, dermis and subcutaneous tissue, and its appendages, *i.e.*, hair, sebaceous glands, sweat glands and arrector muscles (Fig. 1a). The epidermis and dermis are separated by the basement membrane, and the dermis contains numerous blood vessels and nerves (1).

The epidermis is composed of keratinocytes, melanocytes, and immuno-responsive cells (2) (Fig. 1b). Keratinocytes, keratin-forming cells, are the largest cellular component of the epidermis (>90%), which can be further divided into the basal, Malpighian, and horny layers. As keratinocytes move outward from the basal layer to the skin surface (the horny layer), they begin to synthesize the insoluble protein, keratin. Melanocytes, the melanin-forming cells, also are located in the basal layer, and secrete their melanin-pigment granules (melanosomes) into surrounding keratinocytes. Thus, melanocytes are exocrine secretory cells which account for approximately 5 to 10% of epidermal cells. Immunologic cells within the skin, such as Langerhans' cells, are sparse; however, they play a major role in immunologic responses within the skin.

The epidermis performs several major functions in mammals, including: (a) prevention of the penetration of foreign materials and the loss of water from the skin surface; (b) mediation of immunologic responses to foreign materials by epidermal Langerhans' cells; and (c)

pigmentation of the skin (1). The melanin pigments distributed in keratinocytes function primarily to absorb solar irradiation of the skin surface and to reduce the penetration of ultraviolet light (UVL) to the dermis (2).

B. MELANOCYTES

1. Origin, Distribution, and Subpopulations

Rawles, in the late 1940s, was the first to report that melanocytes in vertebrates arise from the neural crest of embryonic tissues (3), and it is now believed that epidermal melanocytes derive embryonically from melanoblasts that originate from neural crest cells of the neural tube. In humans, melanoblasts begin to migrate from this embryonic tissue as early as 15 to 18 days' gestation and their differentiation into melanocytes occurs subsequently within the epidermis by the 6th to 8th week of gestation (4).

The number and distribution of melanocytes in human skin varies among individuals and regions of the body. Racial differences in melanin pigmentation are not due to the difference in the number of melanocytes; rather, they reflect the ability of the melanocytes to synthesize melanosomes and melanin pigments and the pattern of distribution of the pigment granules (5). In general, there are more melanocytes in exposed areas (*e.g.*, forearm) than in non-exposed areas (*e.g.*, buttock); however, exposure to UVL appears to increase the number of functioning melanocytes even in non-exposed skin (6).

Melanocytes can be classified into two subpopulations according to functional differences: (a) secretory melanocytes, in the epidermis and hair follicles, which discharge melanosomes into surrounding cells, and

(b) continent melanocytes, primarily in extracutaneous sites (*e.g.*, the uveal tract and leptomeninges), which do not discharge melanosomes (7). There are structural differences, also; for example, melanosomes synthesized by uveal melanocytes are spherical and of uniform size (about 60 nm in diameter), whereas melanosomes in epidermal melanocytes are ellipsoidal and vary in size (from about 30 x 45 nm to 50 x 100 nm) (8). Fully differentiated melanocytes in the uveal tract contain minimal amounts of endoplasmic reticulum (ER) and Golgi complex, indicating limited activity and production of only small amounts of melanin-pigment granules.

2. Functions of the Epidermal Melanin Unit (EMU)

In humans, pigmentation of the skin is controlled by four major components: (a) oxygenated hemoglobin (red) in capillaries and (b) reduced hemoglobin (blue) in veins, and (c) red-yellow pheomelanin and (d) brown-black eumelanin in the epidermis (2). The differences in skin pigmentation is largely determined by melanin. Melanin pigments are synthesized by cytoplasmic organelles termed melanosomes, which, in the skin, are produced by exocrine melanocytes that possess nerve-cell-like dendrites projecting into surrounding keratinocytes. The melanosomes, which are surrounded by a membrane, are transferred along the dendrites from melanocytes into surrounding cells, and can be distributed singly or aggregated in the keratinocytes. Finally, the melanosomes are removed from the skin with the loss of cornified cells. This close relationship between a melanocyte and its surrounding keratinocytes has been designated as EMU (9) (Fig. 1b).

The EMU serves as a structural and functional unit for various types of melanin pigmentation. Thus its function, in terms of skin pigmentation, can be divided into five biologic processes: (a) synthesis of melanosomes in melanocytes; (b) melanization of melanosomes; (c) transfer of melanosomes into keratinocytes; (d) degradation of melanosomes within keratinocytes; and, finally, (e) removal of melanin pigments and melanosomes with the loss of stratum corneum (5) (Fig. 2). These events are strictly controlled by the fully integrated system; *i.e.*, by regulatory factors produced by both melanocytes and keratinocytes and by their interactions.

However, it is not known how melanocytes and/or keratinocytes control either the transfer of melanosomes from the former to the latter or the pattern of their distribution within keratinocytes.

C. MELANOSOMES

1. Morphogenesis

Melanosomes are specialized secretory organelles within melanocytes. There are two main functional and structural components: the enzyme tyrosinase and the melanosomal structural matrix proteins. These two components produce two types of melanin pigment granules: (a) the eumelanosome which produces brown/black eumelanin, and (b) the pheomelanosome which synthesizes yellow/red pheomelanin. The former granule is an ellipsoidal organelle, whereas the latter is more oval or spherical. The melanocyte in normal skin synthesizes mainly one type of melanin pigment (eumelanin), but the melanocyte in hair follicles can produce either pheomelanin or eumelanin. In certain animals, however, such as agouti mice, the melanocyte can synthesize

both types of melanin, by switching the synthesis from one type of melanosomes to the other during a hair growth cycle (10).

According to Jimbow (11), there are four stages of melanosome maturation in both eumelanogenesis and pheomelanogenesis, as depicted in Figure 3. In stage I, eumelanosomes are spherical vacuoles containing amorphous, proteinaceous material and a few microvesicles called vesiculoglobular bodies. In stage II, the eumelanosomes become ellipsoidal and produce organized filaments (lamellae) associated with microvesicles. In these two stages, no tyrosinase activity is detectable within both types of melanosomes. In stage III, tyrosinase activity becomes detectable and melanization begins, and in stage IV the eumelanosomes are fully melanized. In pheomelanogenesis, the structures of the stage I pheomelanosomes are identical to that of eumelanosomes. Pheomelanosomes are spherical in all four stages of maturation and contain no lamellae or filaments as seen in eumelanosomes; instead, they congregate in numerous vesiculoglobular bodies (microvesicles).

2. Melanosomal Matrix Proteins

Until recent years, melanosomes were thought to consist of melanins and melanoprotein, the latter being a mixture of tyrosinase and melanin pigment. Since the 1970s, however, numerous studies have revealed other melanosomal proteins, in addition to tyrosinase, as components of the structural matrix of melanosomes (12, 13). Furthermore, biochemical studies of melanosomes isolated from murine melanomas (*e.g.*, B16 and HP types) have also identified lipids and other structural matrix proteins (14, 15). The lipids are located mainly on the outer

surface, and the structural proteins form the matrix core and control the structural differentiation of melanosomes (16). By gel electrophoresis [sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)] the melanosomal structural proteins can be separated into at least 15 or 16 polypeptide bands (17). It is interesting that most of them are of similar size but vary in their amount according to the tissue. More important, several bands appear specific to either normal or malignant melanocytes (18). Because it is thought that these melanosomal proteins may represent certain features uniquely expressed in normal or neoplastic melanocytes, they have been used as immunogens to develop monoclonal antibodies (MoAbs) for studying their function in the differentiation and transformation of melanocytes.

D. TYROSINASE

1. Function and Structure

Tyrosinase, a glycoprotein, is the bifunctional, rate-limiting enzyme in melanogenesis: it converts tyrosine to dopa and thence to dopaquinone. Subsequently, dopaquinone is auto-oxidized to give rise to either eumelanin (in the absence of thiol compounds), or pheomelanin [in the presence of thiol compounds, such as cysteine and glutathione (GSH)] (2, 19).

The polypeptide core of tyrosinase is synthesized and glycosylated in rough endoplasmic reticulum (RER) and the Golgi complexes, it then translocates through coated vesicles to "premelanosomes" (immature melanosomes) (20). The frequent occurrence of multiple forms of tyrosinase (21) hindered elucidation of its primary structure, but this has now been established for fungus, bacterium, mouse and human (22-

25). Tyrosinase is a single-chain protein; it possesses melanocyte specificity, transmembrane domains, two potential copper-binding regions and multiple glycosylation sites (26). Its molecular weights range from 30 kDa (bacterium) to 70 kDa (human). Analysis of the amino acid sequence of tyrosinases from *Streptomyces glaucescens*, *Neurospora crassa* and mouse revealed only 8.7% homology; however, there was 38% homology in two stretches of the three tyrosinases, indicating that this region might contain site(s) of activity or the binding site for a copper complex (5). The tyrosinase genes from bacterial, murine, and human melanocytes have been recently cloned (23, 24, 27).

2. The Pigmentation Genes

There has been tremendous interest in cloning of the tyrosinase gene because the enzyme is crucial in regulating the metabolic pathway of melanin formation. The tyrosinase genes at the albino locus (c locus) have been cloned, and mapped to chromosome 7 in mice and chromosome 11 in humans (27, 28). Another tyrosinase-related gene has been mapped to the brown (b) locus on chromosome 4 in mice (29), and more than 50 other loci have been identified that affect melanin pigmentation in mice.

The albino gene in mice and humans is composed of five exons and four introns; it is about 70 kb in mice and greater than 35 kb in humans (30, 31). The albino locus was proposed as the structural locus for tyrosinase because of the lack of pigment generated by mutations at that locus. Recent experiments have demonstrated that the protein encoded by the albino gene functions as tyrosinase. First, when transfected into tyrosinase-deficient cells, the gene generated

tyrosinase activity (32). Second, minigenes containing tyrosinase-coding sequences and suitable regulatory sequences produced transgenic mice which were not fully pigmented, but their pigmentation specifically limited to the melanocytes was significantly increased (32, 33). Finally, an immunoprecipitation study confirmed that the protein encoded by the albino locus functioned catalytically as tyrosinase (34). Thus, it is clear that the protein encoded by the albino locus is tyrosinase and that its function is critical to melanin synthesis. It is still not known, however, whether the product of the albino locus is the only protein functioning as tyrosinase and, if so, how it interacts with other melanogenic proteins [*e.g.*, post-tyrosinase regulatory factors and tyrosinase-related proteins (TRP)].

The b-locus gene has very similar structure and organization to the albino locus gene, and its product protein, termed TRP, has many of the features characteristic of tyrosinase. The b-locus gene in mice is about 18 kb and is composed of 8 exons and 7 introns (35). The human b-locus gene was recently identified and cloned (36). Although the specific function of the protein encoded by the b-locus gene is not clear, a mutation at this locus causes the production of brown rather than black pigment (37). It was recently shown that the b-locus protein possesses catalase activity (38) and there are indications that it might also contain 5,6-dihydroxyindole (DHI) conversion factor (39), but the substrates and products of reactions that may be catalyzed by the b-locus protein have not been identified.

E. MELANINS

1. Eumelanin

Brown-black eumelanin is present in normal skin and hair. It is derived from tyrosine and composed mainly of monomer units of 5,6-DHI, is insoluble in acid and alkali, and contains 6 to 9% carboxylic nitrogen and 0 to 1% sulfur (Table 1). The insolubility of this pigment hinders the study of its chemical structure and physical properties, as eumelanin can be solubilized only by extensive treatment with chemicals such as concentrated hydrochloric acid (40). Even so, the chemical structure of eumelanin has been determined through chemical degradation studies, which have revealed small amounts of pyrrolic acids, mainly tricarboxylic acid 2 (41). In addition, the study by Nicolaus and Piatelli (42) identified trace amounts of 5,6-DHI and 5,6-dihydroxylindole-2-carboxylic acid (DHICA) among products formed by alkaline fusion of eumelanin. However, the low yields of these products has precluded assessment of their significance as components of the main structure of eumelanin.

Overall, the natural eumelanin pigment is thought to be a highly heterogeneous polymer consisting mainly of 5,6-DHI units derived from tyrosine and pyrrolic acid residues (41). However, neither the sequence nor the proportion of these units in eumelanin, nor the average molecular weight of this pigment, has been defined.

2. Pheomelanin

Unlike eumelanin, red-yellow pheomelanin is present mainly in hair follicles. It is soluble in alkali and contains a high concentration of sulfur (9-12%) (Table 1). It derives from tyrosine by the pathway common to

eumelanin synthesis, but requires the intervention of the thiol compounds, cysteine and GSH. Because of its solubility in dilute alkali, pheomelanin can be investigated with column chromatography (43); for example, pheomelanin pigments from the feathers of New Hampshire hens were separated into four major subfractions (gallophaeomelanins-1, 2, 3 and 4), all containing sulfur and some also containing proteins. However, these fractions were mixtures of similar pigments and did not contain well-defined chemical entities. Importantly, when oxidized with hydrogen peroxide, pheomelanin gave rise to cysteinic acid and small amounts of glycine and aspartic acid, but no pyrrole or indole derivatives—the characteristic degradation products of eumelanin (44). These findings thus indicate no structural similarity of pheomelanin and eumelanin. In addition, the study by Fattorusso *et al.* (45) indicated that pheomelanin pigments from some animals (*e.g.*, rabbit and goat), red-haired humans, and certain human melanomas were similar in structure but differed in molecular size, the proportion of certain units in their polymeric chain, and the degree of attachment to proteins. As with eumelanin, the molecular structure and weight of pheomelanin are unknown.

3. Mixed-type Melanin

Evidence is accumulating that natural melanin in the melanocyte is a co-polymer (mixed-type melanin) of eumelanin and pheomelanin and that the 'pure' eumelanin or 'pure' pheomelanin seldom, if ever, exists in mammalian tissues (12), including human skin (46).

F. MELANOGENESIS

1. The Classic Pathway of Eumelanin Synthesis

In late 1895, Bourquelot and Bertrand (47) reported that a crystalline substance isolated from a toadstool turned red and eventually gave rise to a black precipitate when brought into contact with an enzyme they had found in the same toadstool. This pigment-forming enzyme was later identified as tyrosinase and subsequently found in many other plants and tissues of invertebrates (19). In 1950, Fitzpatrick *et al.* (48) established the presence of tyrosinase in melanocytes of normal human skin.

The formation of melanin from tyrosine catalyzed by tyrosinase is accompanied by changes in color, in which red appears first, then purple, and finally black. Raper (49) was able to identify dopa, DHI, and a small amount of DHICA, by stopping the oxidation at an early stage of the reaction and allowing the red solution to decolorize in the absence of oxygen. When exposed to air for auto-oxidation, DHI darkened rapidly and deposited a black precipitate, whereas the DHICA darkened slowly and did not deposit black pigment. Thus, Raper (49) proposed a metabolic pathway for the early stages of the eumelanin synthesis; later, Mason *et al.* (50) confirmed and partly modified this pathway, as shown in Figure 4.

The Raper–Mason pathway is still generally accepted as the pathway of eumelanin synthesis. In the initial step, tyrosinase catalyses tyrosine to dopa and thence to dopaquinone, which is highly reactive. Dopaquinone undergoes intramolecular changes and gives rise to dopachrome, which is quickly oxidized; this gives rise to DHI, which is converted to eumelanin through the intermediate 5,6-indolequinone

(51). Although the Raper–Mason scheme of melanin synthesis represents a milestone in pigment-cell research, it may be very different from the true situation *in vivo*, especially in humans. In addition, various other factors can affect the synthetic pathway of melanin in humans; these are discussed below.

2. The 'New' Pathway of Pheomelanin Synthesis

It is now well established that eumelanin and pheomelanin arise from a common metabolic pathway which bifurcates after the formation of dopaquinone. The fate of dopaquinone appears to depend largely upon the biochemical environment of the melanocyte: in eumelanin-forming melanocytes, dopaquinone uses the Raper–Mason pathway to give rise to a black insoluble eumelanin; in pheomelanin-forming melanocytes, thiol compounds (*e.g.*, cysteine, GSH) create an alternative pathway, which leads to the synthesis of yellow-red pheomelanin through the intermediate, cysteinyldopa (CD). Thus, the ability to switch from eumelanogenesis to pheomelanogenesis appears to be associated with interactions between the thiol compounds and dopaquinone (52).

It used to be thought that sulfhydryl compounds, such as glutathione (GSH), inhibited melanin pigmentation and that, therefore, melanin could not be synthesized even when both tyrosine and active tyrosinase existed in the epidermal melanocytes (53). It was further postulated that melanogenic stimuli such as UVL could oxidize or destroy these sulfhydryl compounds and remove the inhibition (54). However, numerous studies have now shown that the action of sulfhydryl compounds is not related to inhibition of tyrosinase but to its own ability to react with dopaquinone (55). It has been suggested that the

formation of CDs may result from the direct addition of cysteine to dopaquinone, or from interaction between dopaquinone and GSH followed by hydrolysis of the resulting glutathionedopa adducts, as shown in Figure 4 (56). However, it is not clear which of these pathways prevails *in vivo*; this is because the distribution of cysteine and GSH within melanocytes is unknown. Unless there is a specific mechanism to translocate cysteine into melanosomes, GSH may well be the major source of the thiol compound for the production of pheomelanin (57). The major products of interaction between the thiol compounds and the quinone system are 5-S-CD and 2-S-CD, the major precursors of pheomelanin (11).

As the addition of thiol compounds to dopaquinone is not under enzymatic control, the switch in melanogenesis must be associated with regulation of the amount and distribution of sulfhydryl compounds in melanocytes (57). This view was supported by the findings in a recent study by Benedetto *et al.* (58) in tortoiseshell guinea-pig skin of various colors and in skin of black and yellow mice. As expected, the lowest and highest degree of activity of GSH-reductase was observed in eumelanin-producing melanocytes and in the pheomelanin-producing melanocytes respectively. In addition, the tissue level of GSH was low in the skin of black C57 mice but high in the skin of yellow agouti mice (52). These results may also explain an earlier observation that pheomelanin-forming melanocytes from the hair follicles of agouti mice produced black pigment initially but changed to yellow pigment when enough GSH or cysteine was added to the culture medium (59). *In vivo*, the amount of GSH needed for this switch appears to depend upon the genetic background of the animals (58).

3. Regulation of Melanin Synthesis

a. In the Cells

The pattern of migration of melanocytes from the embryonic neural crest is under genetic control and thus can lead to interesting phenomena when the distribution in skin is not uniform, as in zebras and giraffes (37). Melanocytes are also influenced by extracellular factors that determine not only the amount of melanin synthesized but also the type of melanin produced. Several studies have demonstrated that a single dose of UVL irradiation increases melanin synthesis but does not necessarily increase the number of melanocytes. This indicates increased activity of tyrosinase, but that repeated UVL exposures increase both the tyrosinase activity and the number of functioning melanocytes (*see* review in ref. 60). This reaction occurs not only in the exposed skin but also in that person's unexposed skin. Another melanogenic stimulus is melanocyte-stimulating hormone (MSH), a peptide produced by the posterior lobe of the pituitary gland. After binding to melanocyte surface receptors, MSH can enhance eumelanin synthesis by increasing intracellular cAMP and thereby elevating tyrosinase activity (61). Furthermore, the melanocyte functions in the EMU by responding to various signals produced by the melanocyte itself, as well as from the keratinocyte and Langerhans' cell (62-65).

b. In the Organelles

Melanogenesis is regulated at the organelle level also, leading to wide variations in the number, size, and distribution of melanosomes (66). As shown in Table 1, the eumelanosome is ellipsoidal and contains lamellar structures (or filaments), whereas the pheomelanosome is

spherical and contains microvesicles (vesiculoglobular bodies). The synthesis and assembly of structural proteins for both types of melanosomes are under genetic control and may be altered in pigmentary diseases that give rise to abnormal pigmentation (14). The pattern of distribution of melanosomes in keratinocytes, also, is under genetic control and responsible for the variety of skin colors in humans. A recent study of the glycosylation inhibition of tyrosinase indicated that ER, Golgi complexes, lysosomes and coated vesicles (including microvesicles or vesiculoglobular bodies) participate directly or indirectly in the synthesis, translocation, and assembly of tyrosinase and structural proteins (67).

c. Along the Metabolic Pathway

Melanin synthesis is regulated at the level of the metabolic pathway also. The amount of melanin synthesized can be increased or decreased, as in albinism (melanin pigments decreased or absent), or its type changed, as in the reversible switch from black eumelanin to yellow pheomelanin (5). As tyrosinase is a rate-limiting enzyme, the activation and inhibition of tyrosinase is critical in melanin formation. The modification (*e.g.*, glycosylation) and delivery of tyrosinase from Golgi complex to melanosomes also affects the enzymic activity. In addition, dopachrome-conversion factor, indole-conversion factor, and indole-blocking factor have been described as post-tyrosinase regulatory factors in melanogenesis. These factors appear specifically to affect the post-tyrosinase stages (8). It was indicated recently that intracellular GSH is requisite to the formation of pheomelanin, converting part of dopaquinone to glutathionedopa, which cannot give rise to pheomelanin

unless it is further oxidized to cysteinyldopa (55). If the further oxidative step is absent, the formation of glutathionedopa would only 'side-track' the dopaquinone and produce neither pheomelanin nor eumelanin. Thus, intracellular GSH is critical in regulation of the switch of melanogenesis; *i.e.*, from eumelanogenesis to pheomelanogenesis.

G. MoAbs IN THE STUDY OF MELANOGENESIS

With establishment of the MoAb technique, the MoAbs against specific proteins that take part in melanogenesis have proven useful in the investigation of pigmentation of the skin (Table 2). Since the early 1980s, numerous MoAbs have been described to recognize components of the pathway of melanin biosynthesis, including melanosomal proteins (68) and the glycoprotein tyrosinase (69).

The subcellular distribution of tyrosinase has been delineated in immunocytochemistry studies with anti-tyrosinase MoAb (69). A high level of tyrosinase was found in melanosomes, the Golgi–ER–lysosome (GERL) system and coated vesicles, a finding that advanced the understanding of the biosynthesis of tyrosinase and the initial step of the pathway of melanin synthesis. Other MoAbs have been developed to recognize proteins involved in melanosome biosynthesis; for example, four MoAbs against human melanosome-specific antigen (HMSA) have been established in this laboratory by immunizing mice with the purified melanosomal fractions from human malignant melanomas removed at autopsy (68, 70, 71). The immunogen was non-melanized protein consisting of at least 7 or 8 major polypeptide bands under SDS-PAGE. The four MoAbs, designated HMSA-1, 2, 3 and 4, reacted with neoplastic melanocytes from melanocytic nevi and malignant

melanomas including amelanotic lesions. None of them reacted with normal melanocytes from non-melanocytic tumors or with normal tissues from fetuses and adults. Thus, these MoAbs appear to identify antigens associated with melanosome differentiation in neoplastic melanocytes, thereby enabling immunohistologic diagnosis of melanocytic lesions. Moreover, in a study with immuno-electron microscopy, MoAb HMSA-1 identified cytoplasmic components of melanoma cells that were located in the inner matrix of melanosomes and vacuolar structures (probably stage 1 melanosomes) and the smooth endoplasmic reticulum (SER) but not in the Golgi complex and coated vesicles as in the tyrosinase study (72). This observation supports the postulate that melanosomal protein and tyrosinase are synthesized at different sites. Although these antibodies have provided specific tools for studying human pigmentation and various pigmentary diseases or tumors, so far they have failed to provide information about the synthetic pathway of melanin pigment itself.

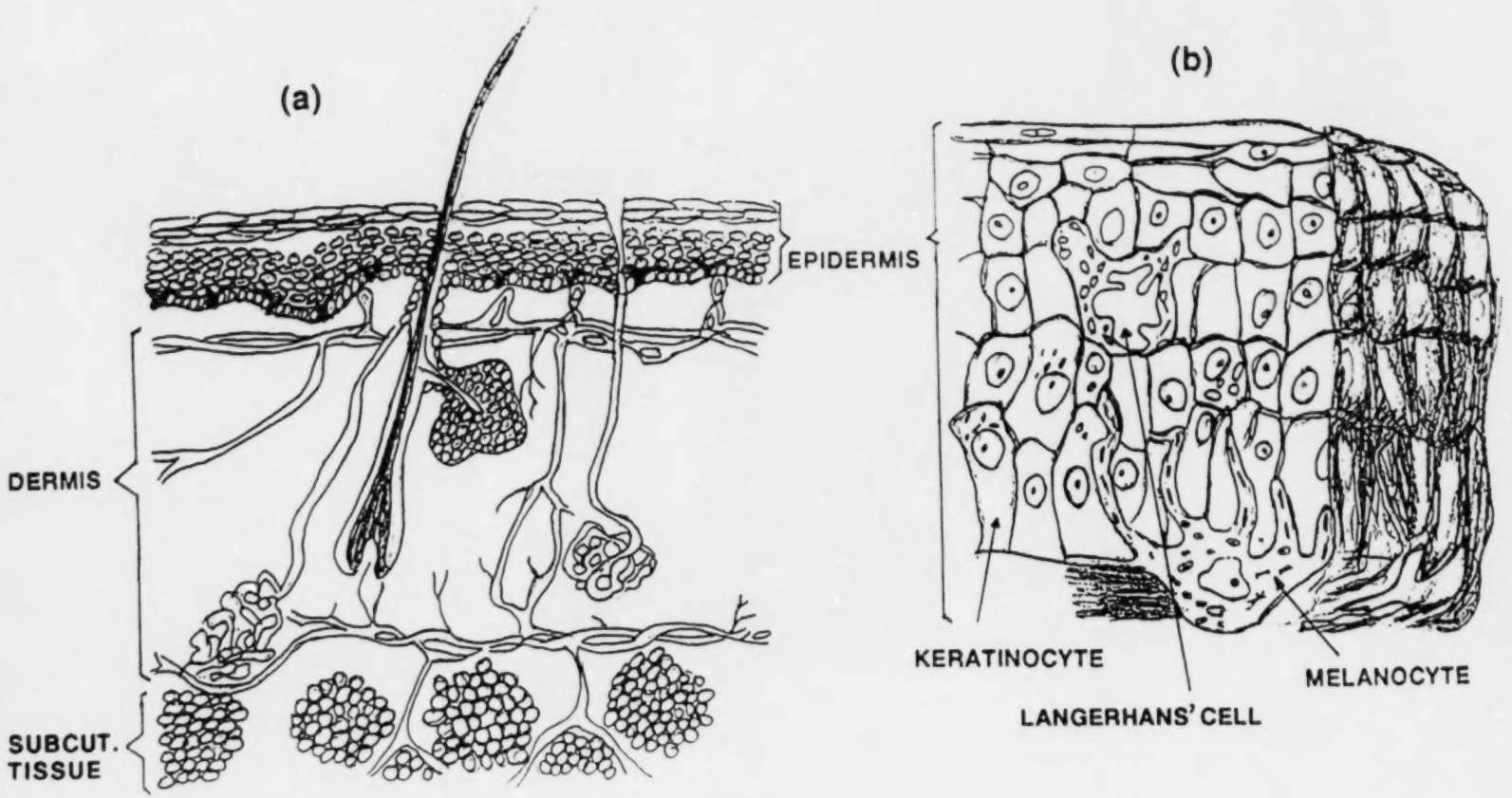


Figure 1
a) Structure of Skin, b) Cell Types of Epidermis and Epidermal Melanin Unit (EMU), Consisting of a Melanocyte and Neighboring Keratinocytes (adapted from ref. 1 and ref. 2)

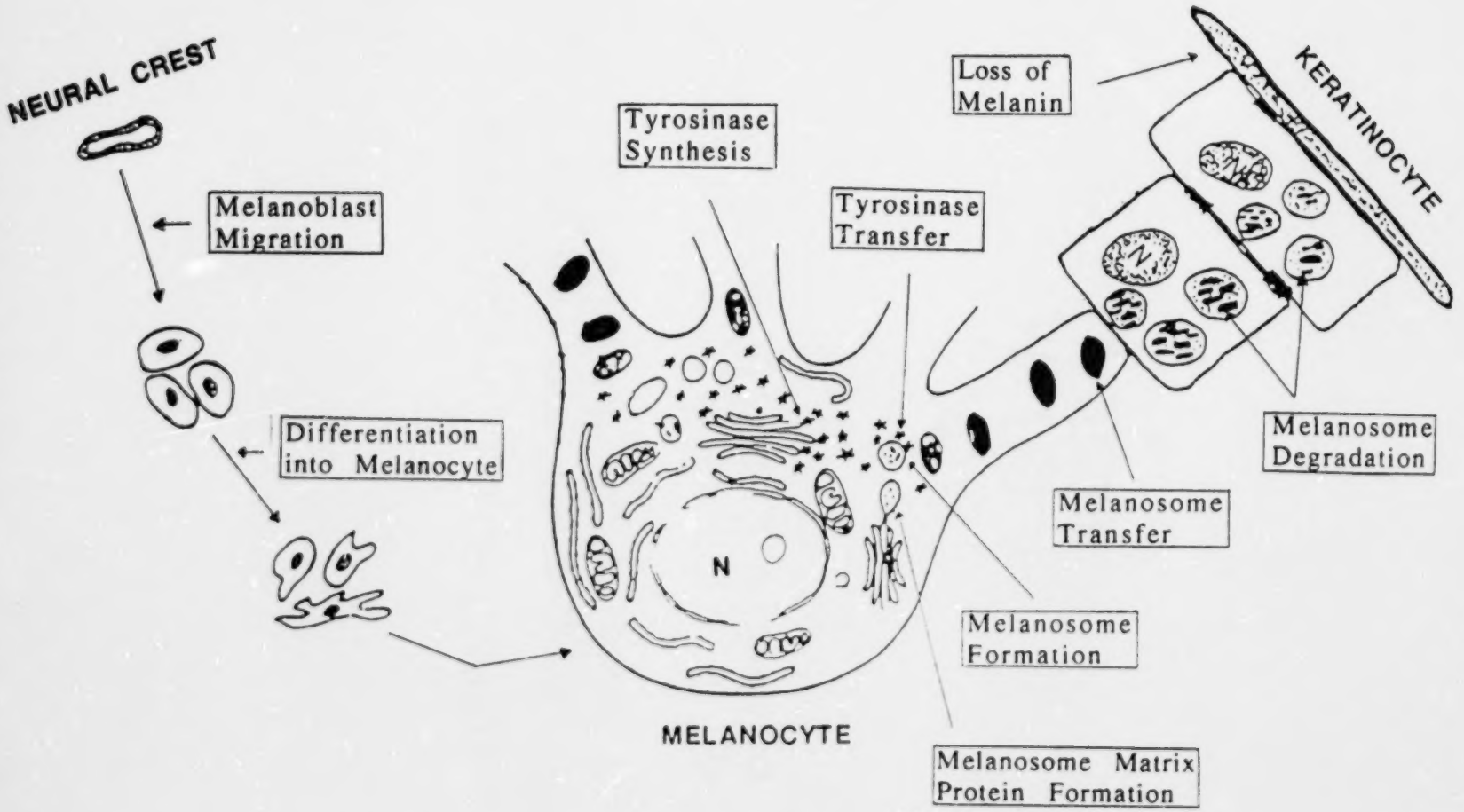


Figure 2
Origin and Differentiation of Melanocytes (adapted from ref. 5)

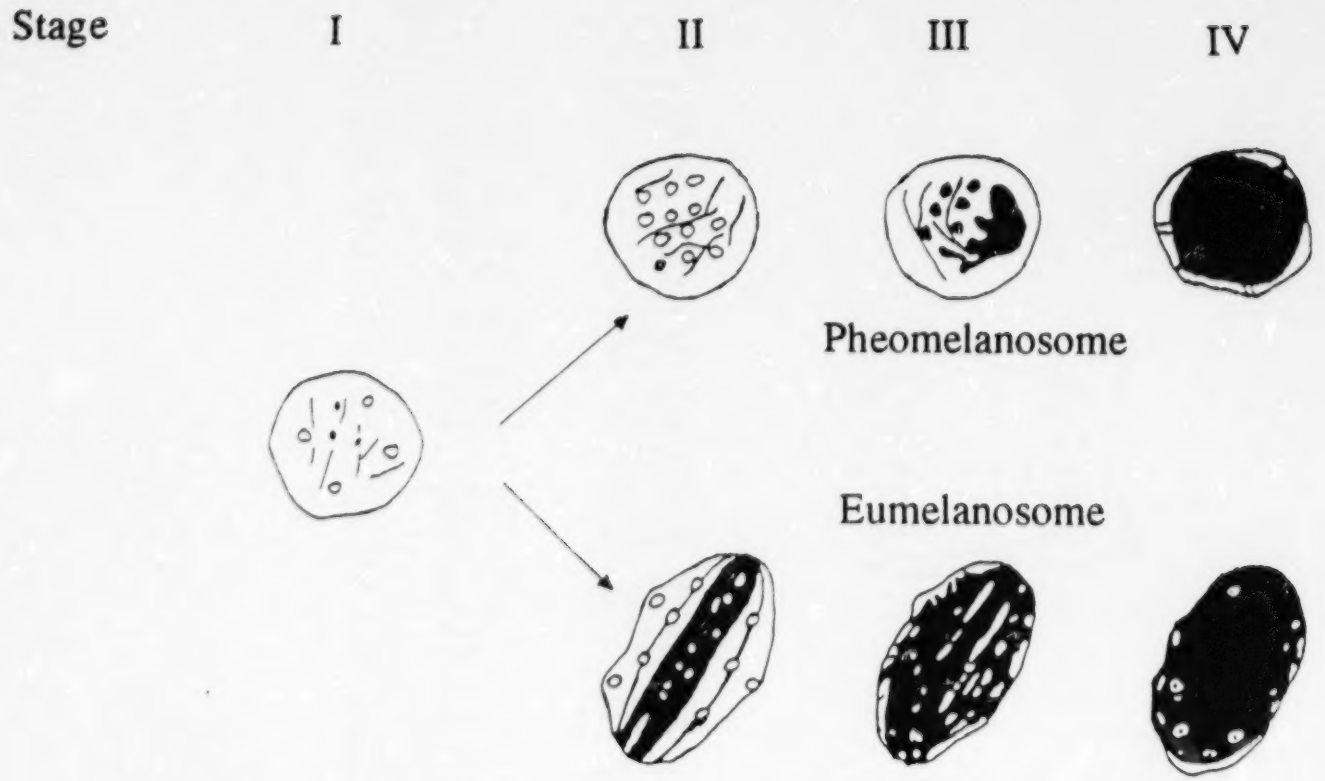


Figure 3
Differentiation of Eumelanosomes and Pheomelanosomes
(adapted from ref. 11)

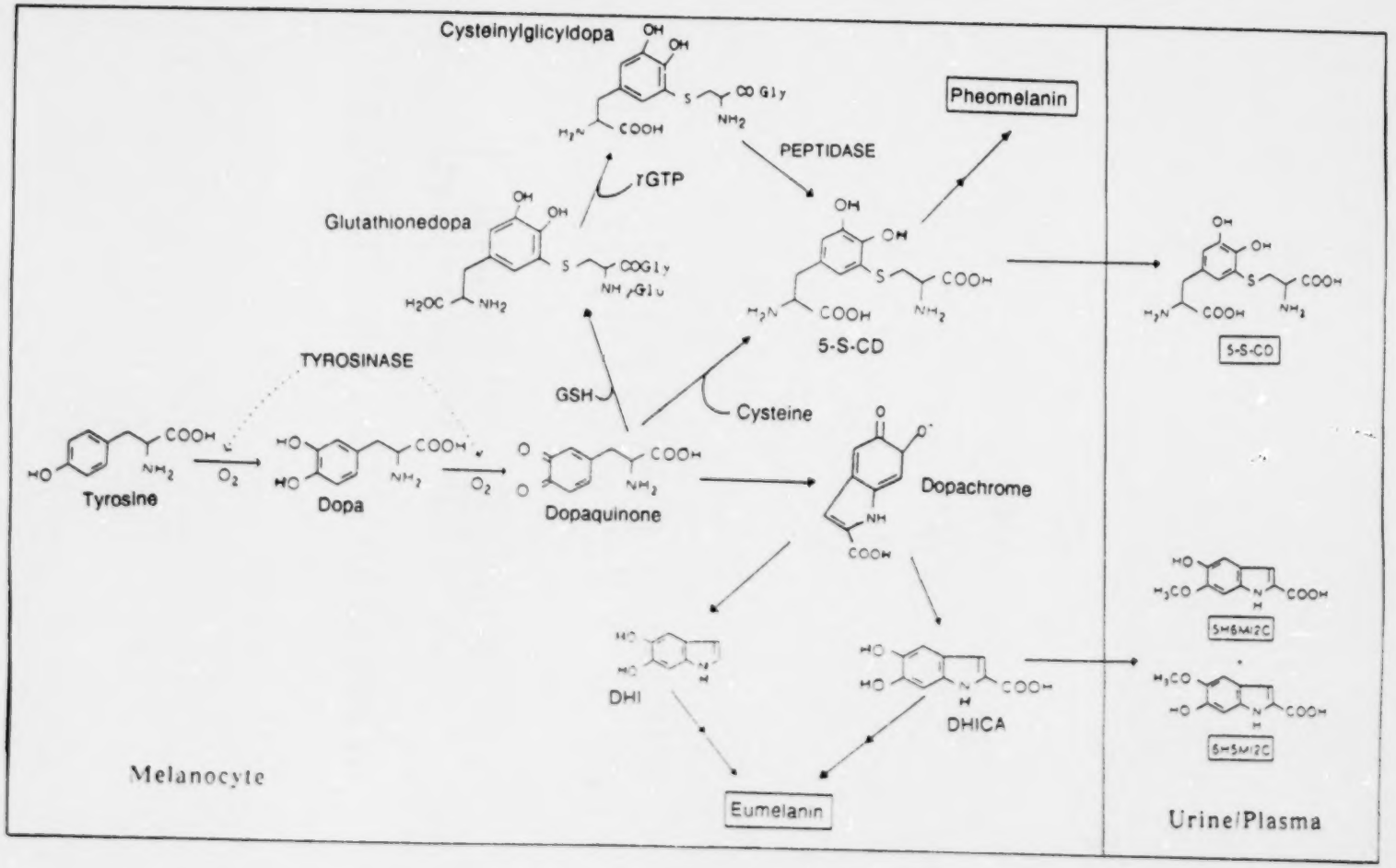


Figure 4
Biosynthesis of Eumelanin and Pheomelanin and Their Metabolites in Urine and Plasma (adapted from ref. 5)

Table 1 Comparison of Eumelanogenesis and Pheomelanogenesis in Humans

	Eumelanogenesis	Pheomelanogenesis
Melanosome	Eumelanosome	Pheomelanosome
Location	Skin & hair	Hair (mainly)
Shape	Ellipsoidal	Oval or spherical
Matrix structure	Lamellae or filaments	Microvesicles
Melanin	Eumelanin	Pheomelanin
Color	Brown/black	Red/yellow
Solubility	Insoluble in acid & alkali	Soluble in alkali
Sulfur	0 to 1%	9 to 12%
Nitrogen	6 to 9%	8 to 10%
Structure		
Chemical	5,6-DHI (mainly)	Benzothiazine
ESR[1]	Single, structure-less spectrum	High-, central-, and low-field components
Precursor	5,6-DHI	5-S-CD
Analysis	PTCA[2]	AHP[3]

1. ESR: electron spin resonance, from ref. 73
2. PTCA: pyrrole-2,3,5-tricarboxylic acid, from ref. 74
3. AHP: aminohydroxyphenylalanine, from ref. 74

Table 2 Characteristics of Major MoAbs Used in the Study of Melanogenesis

MoAb*	Immunogen	Antigen Type	Specificity	Ref.
HMSA-1	Melanosomes of human metastatic melanoma solubilized & purified	Melanosomal protein (65 & 35 kDa)	Melanoma & nevus cells	68
HMSA-2	Melanosomes of human metastatic melanoma solubilized & purifified	Melanosomal protein (53, 28, 23, 18.5 kDa)	Melanoma & nevus cells	70
NKI/C-3	Plasma membrane of MeWo melanoma cells	Glycoprotein (25 to 110 kDa)	Melanoma & nevus cells	75
2G10	Highly pigmented human melanoma cells	T4 tyrosinase (75 kDa glycoprotein)	Melanocytes & melanoma cells	76
5C12	Partly purified tyrosinase of human melanoma cells	Tyrosinase (61 kDa)	Melanocytes & Melanoma cells	77
1C11	MM96L cells (mixture of live & methanol-fixed cells)	Glycoprotein (37.5-47 kDa)	Melanoma cells	78
TMH-1 & TMH-2	T4-tyrosinase (isolated & purified from melanosomes of murine melanoma cells)	A protein encoded by b-locus	Murine melanoma cells	79
HMSA-5 /TA 99	Purified melanosomes of normal melanocytes	Glycoprotein (69 to 75 kDa)	Melanocytes & melanoma cells	129, 130
ME491, 492 & LS 62	Partly purified melanosome preparation from SK-23 cells	MAG (30 to 60 kDa)	Melanoma & nevus cells	134

*All except TMH-1 & TMH-2 are specific to human tissue.

CHAPTER 2
RESEARCH PLAN

A. RATIONALE

As presented previously, knowledge of the formation of melanin is still incomplete. For example, little is known about the subcellular distribution of melanin precursors, such as 5-S-CD, and various intermediates (*e.g.*, cysteine and GSH), or even the pathway for synthesis of melanin itself within the melanocytes. We know that tyrosinase is the main enzyme in the formation of melanin, but have little understanding of factors regulating the post-tyrosinase metabolic pathway for both eumelanogenesis and pheomelanogenesis, directly or indirectly. Furthermore, most melanocytic diseases, such as vitiligo and albinism, and certain types of skin cancers (*e.g.*, malignant melanoma), are accompanied by abnormal melanin synthesis, but the triggers and mechanisms for this have not been clarified.

A major precursor of pheomelanin, 5-S-CD, is released into the urine of many melanoma patients, and melanin metabolites are present in the urine (melanuria or melanogenuria) of patients who have advanced-stage melanoma (80, 81). The plasma level of 5-S-CD in melanoma patients without metastasis is twice that in healthy persons, and it is 7- to 45-times normal in patients with extra-regional metastasis (82). Interestingly, 5-S-CD is also released into the urine of healthy persons following exposure to sun or UVL, but the mechanism of this release has not yet been determined (83). In general, 5-S-CD may have important roles in biological systems other than simply as an intermediate product of pheomelanin synthesis (57).

B. Hypothesis

A pheomelanin precursor, 5-S-CD (mol. wt. 316), when coupled with a large protein carrier, can be used to elicit a specific antibody response in mice. Further, this new antibody can be used to study melanogenesis and to detect occult melanomas, based upon the plasma/urine levels of 5-S-CD in melanoma patients with and without metastasis.

C. Objective

To develop a MoAb against the pheomelanin precursor 5-S-CD and to characterize its specificity and reactivity.

D. Aims

a). To establish histopathologic marker(s) useful for identifying abnormal melanin pigmentation (*e.g.*, pheomelanin synthesis) of pathologic tissues in melanocytic diseases or tumors.

b). To develop a radioimmunoassay (RIA) for the detection of occult melanoma.

c). To identify subcellular organelles that participate directly or indirectly in the biosynthesis of pheomelanin.

F. Experimental Design

The study consisted of four series of experiments, as follows.

Chapter 3 describes the preparation of immunogens. The hapten 5-S-CD was coupled to large protein molecules, such as keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), or poly-L-lysine. Two bifunctional reagents, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and glutaraldehyde, were employed for the coupling reaction. The

immunogens and the screening antigens were obtained from different coupling reactions with high coupling efficiency.

Chapter 4 deals with the immunization of mice with the 5-S-CD–carrier conjugate. The antigen 5-S-CD, coupled to various protein carriers, was injected into BALB/c mice. Complete Freund's adjuvant was used for the first injection and incomplete Freund's adjuvant was used for the remaining injections. The efficiency of immunization was assessed by ELISA of the immunized mouse sera and immuno-staining of cultured cells; the mice with the highest antibody titer and positive reactivity with melanoma cells were chosen for the cell fusion experiments.

Chapter 5 discusses the hybridoma development. Cell fusions were carried out with the fusogen polyethylene glycol (PEG) 4000. Hybridoma cells were screened by ELISA and staining of cultured cells and positive hybridomas were subcloned.

Chapter 6 describes characterization of the new MoAb 6D4. Immunoassays were used, such as the sub-isotyping method, together with indirect immunoperoxidase staining and the competitive ELISA. These methods were employed to characterize the subclass, the specificity, and the reactivity of MoAb 6D4 with melanomal tissues.

Chapter 7 summarizes the studies and discusses potential clinical applications of MoAb 6D4 and the potential future directions of this research.

CHAPTER 3
PREPARATION OF IMMUNOGENS

A. INTRODUCTION

A hapten, such as 5-S-CD [mol. wt. 316; (84)], is an antigen with a small or single antigenic determinant which is the site for an antibody to bind in the antigen–antibody reaction (85). Although a hapten can bind to an antibody, it is unable to elicit a strong immunologic response by itself (86). However, if the hapten (in our case 5-S-CD) is coupled to a protein molecule, such as bovine serum albumin (BSA), this 5-S-CD–BSA conjugate is able to stimulate strong antibody production (87). The protein molecule (the carrier) renders its coupled hapten capable of eliciting a strong antibody response. As 5-S-CD cannot be linked to a protein carrier directly, the hapten–carrier coupling reaction requires coupling reagents, such as carbodiimide (88) and glutaraldehyde (89), as shown in Figure 5. In addition, the choice of coupling procedure is crucial, because the antigenic activity of a hapten may be affected by different conjugation procedures (90); this is discussed later in this chapter. In this study, three coupling methods and two coupling reagents (glutaraldehyde and carbodiimide) were chosen, based on the chemical structure of 5-S-CD, to prepare the immunogen (5-S-CD–protein carrier conjugate) for immunization. In addition, two methods (HPLC and UV absorbance) were used to examine the efficiency of the coupling methods.

B. Materials and Methods

1. Chemicals

Pheomelanin and 5-S-CD were synthesized by Ito's methods (84, 91). Briefly, 0.05 M sodium phosphate buffer (pH 6.8) containing 0.5 mM L-dopa and 1.0 mM L-cysteine was stirred vigorously at room temperature with mushroom tyrosinase (3.6 mg/mL, 2750 U/mg) added. The incubation was continued for 30 to 45 min for 5-S-CD and for 3 to 4 h for pheomelanin. For 5-S-CD, the incubation was monitored at 255 and 293 nm by UV absorbance of aliquots. The end-product of the reaction for pheomelanin was a dark red precipitate; this was washed with 1% acetic acid (v/v) and acetone, dried over P_2O_5 and NaOH in a desiccator, and left in air to equilibrate with moisture for 24 h. The eumelanin was kindly supplied by Dr S Ito (Toyoake, Japan). Keyhole limpet hemocyanin (KLH), BSA, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), glutaraldehyde, citraconic anhydride, trichloroacetic acid (TCA) and poly-L-lysine were purchased from Gibco Bethesda Research Laboratory (Burlington, Ont.) and Sigma Chemical Company (St Louis, MO, USA).

2. Coupling Method

a. Coupling with EDC

1). Modification of Goodfriend's Method

The coupling of 5-S-CD and KLH or BSA was carried out with EDC by a modification of Goodfriend's method (88). First, 4 mg of 5-S-CD and 40 mg of EDC were dissolved in 2 mL of distilled water. This mixture was incubated for 2 min at pH 5.0 and then for 5 min at pH 8.0 at room temperature, the pH being adjusted with 0.1 N HCl and 0.1 N NaOH.

Then, 2 mL of distilled water containing KLH or BSA was added and the incubation was continued for 3 to 4 h at 4°C. The molar ratio of protein:hapten was approximately 1:20 and the EDC concentration was 10 mg/mL; 100 mM sodium acetate (pH 4.2, adjusted by adding 2 N HCl) was added, and the incubation was continued for another 1 h at 4°C to stop the coupling reaction. Finally, the mixture was dialyzed against 4 changes of 0.01 M modified PBS, pH 7.2 (1 liter 0.1 M stock PBS, 80 g NaCl, 2 g KCl, and 14.4 g Na_2PO_4) with 4 changes in 24 h. The conjugate was stored at 4°C.

2). Modification of Mueller's Method

Coupling of 5-S-CD with BSA or poly-L-lysine was carried out with EDC added, by a modification of Mueller's method (92). The 5-S-CD was first dissolved in boric acid–borax buffer (50 mL of 0.2 M boric acid and 22.5 mL of 0.05 M borax), pH 8.7. Citraconic anhydride was added, to a hapten:citraconic anhydride ratio of 1:10 M. The pH was adjusted continually from 8.5 to 9.0 with 1 N NaOH at room temperature. When the pH was stable, 0.1-mL aliquots of citraconic anhydride were added until this caused no change in the pH; *i.e.*, the reaction was complete. With gentle stirring throughout, the mixture was incubated for 1 h at room temperature and then EDC was added to a final concentration of 10 mg/mL. After 5 to 10 min the carrier protein was added, to a final protein carrier:hapten molar ratio of 1:20 or 1:40. This mixture was incubated for 4 h at 4°C, and dialyzed against 1 Liter of 5% acetic acid (v/v) for 3 h and then against 4 changes of 0.01 M PBS (pH 7.2) for 24 h at 4°C. Finally, the conjugate was stored at 4°C.

b. Coupling with Glutaraldehyde

The 5-S-CD solution (2 mg/mL) was prepared in 0.01 M modified PBS, pH 7.2. The protein carrier (BSA or poly-L-lysine) was added to the 5-S-CD solution at a molar ratio of protein carrier:hapten of 1:20 or 1:40. The glutaraldehyde solution [0.1% or 0.2% (w/v)] in 0.01 M PBS, pH 7.2, was prepared just before use; it was added to the mixture of 5-S-CD and a protein carrier with constant stirring at 4°C, to a final glutaraldehyde concentration of 0.05% or 0.1% (w/v), respectively. The final 5-S-CD concentration was 1 mg/mL. The mixture was incubated for 4 h at 4°C, and dialyzed against 4 Liters of 2% glycine (w/v) for 12 h to stop the reaction. The conjugate was then dialyzed overnight against 4 Liters of 0.01 M modified PBS, pH 7.2, at 4°C (4 changes). Finally, the conjugate was stored at 4°C.

3. Coupling Efficiency

a. High-performance Liquid Chromotography (HPLC)

Aliquots (0.1 mL) of the 5-S-CD-protein mixtures from before and after the coupling reaction were treated with 20% TCA (w/v) for 30 min on ice, then centrifuged for 15 min at 14,000 rpm (6000 *g*) to precipitate the protein. The supernatant fluid (0.01 mL) from each mixture was collected and injected into the HPLC aperture (Waters model 600E; Millipore Corp., Milford, MA, USA); the electrochemical detector (Waters model 460) was set at 750 mV at 25°C. The mobile phase, per Liter, consisted of 12 g of phosphoric acid, 10 g of methanesulfonic acid, and 0.1 mmol of acetic acid in water; the flow rate was 0.7 mL/min and the pH was adjusted to 3.1 with 2 N sodium

hydroxide (93). Finally, the concentration of free 5-S-CD from each sample was calculated from the height of the 5-S-CD peak.

b. UV Absorbance

Two control solutions of BSA (5 mg/mL) and poly-L-lysine (4.5 mg/mL) were prepared in 0.01 M modified PBS solution, pH 7.2. The protein concentration in the control solution was equal to its concentration in the hapten/carrier conjugates. The UV absorbance from each 1-mL sample was read at 280 nm in a DU-65 spectrophotometer (Beckman Instruments, Fullerton, CA, USA), with the background reading set at 0.01 M modified PBS. For readings exceeding 1.0, samples were diluted by the same modified PBS until a valid reading was obtained. Finally, the UV absorbance value of each 1-mL sample was scanned in the spectrophotometer at wavelengths from 200 nm to 400 nm.

C. RESULTS

1. HPLC

In experiments with HPLC, the concentrations of free 5-S-CD from the reaction mixture before and after the coupling reaction were determined according to heights of the 5-S-CD peak (Fig. 6), which usually appeared 12 min after the injection. The 5-S-CD dissolved in 0.01 M PBS (0.5 mg/mL) was used as a control. The concentration of free 5-S-CD in the supernatant fluid before and after coupling was 191 µg/mL and 9 µg/mL, respectively; *i.e.*, most of the 5-S-CD was coupled to the protein carrier, KLH. The same efficiency was obtained in 5-S-CD–BSA coupling experiments, in which the concentrations of free 5-S-CD in

non-coupled and coupled samples were 187 μg/mL and 6 μg/mL, respectively.

2. UV Absorbance

With the BSA concentrations in the BSA solution and the conjugate equal (0.83 mg/mL), the UV absorbance values at 280 nm for BSA and the 5-S-CD–BSA conjugate coupled with the glutaraldehyde method were 0.804 and 1.773, respectively (Fig. 7a). This result clearly shows that the UV absorbance of the conjugate increased dramatically after the coupling reaction and indicated that 5-S-CD was coupled to the protein carrier BSA.

Scanning the 5-S-CD-poly-L-lysine conjugate and the carrier poly-L-lysine gave UV absorbance values of 0.091 for carrier poly-L-lysine alone and 1.567 for the conjugate at 280 nm (Fig. 7b); the poly-L-lysine concentrations alone and in the conjugate were the same (1.13 mg/mL). The result clearly shows that the conjugate coupled with glutaraldehyde gave a much higher UV absorbance value, indicating that coupling efficiency by this method was high.

The UV absorbance value of the conjugate of 5-S-CD and poly-L-lysine coupled by EDC in the presence of citraconic anhydride was greater than with the poly-L-lysine alone (Fig. 7c). The UV absorbance value at 280 nm was 0.646 for the 5-S-CD–poly-L-lysine conjugate, but 0.009 for the carrier (poly-L-lysine) alone. However, compared with the efficiency obtained by the glutaraldehyde method as assessed by the UV absorbance value, the coupling efficiency of this method was low.

D. DISCUSSION

1. Basic Concepts for Hapten–Protein Conjugations

As mentioned previously, hapten 5-S-CD cannot stimulate strong production of antibody: this requires co-operation between antigen-presenting cells (APC) and helper T cells (TH), and between B cells and TH cells (94). This co-operation is mediated by the class II protein of the major histocompatibility complex (MHC), the receptors on the surface of TH and B cells. The antigen is initially processed by APCs, which retain processed antigenic fragments of the antigen on their surface. The TH cells recognize the combination of processed antigen and MHC class II protein via their surface receptors and then help B cells to recognize the antigenic determinant of the antigen by the surface receptors (immunoglobulin) of B cells. Finally, B cells are stimulated to proliferate and divide into antibody-secreting cells (95). If a hapten does not have a binding site for class II protein and the T-cell receptor, it cannot elicit strong production of an antibody. However, this particular site can be added to a hapten by coupling it to a large protein molecule which does possess the site for binding the class II protein and T-cell receptor (96). Therefore, many chemical compounds, such as 5-S-CD, can be used to elicit strong antibody responses by linking them to a protein carrier in which the hapten itself serves as an antigenic determinant for binding to the antibodies on the B cell surface (97). Usually, haptens are coupled to soluble protein carriers, such as BSA and KLH (87).

Some haptens, including 5-S-CD, cannot be coupled directly to carriers; in such cases, coupling reagents are required to perform chemical activation or to be a bridge for the haptens (92). The coupling reagents themselves either become incorporated into the final conjugate

(bridging) or activate certain reactive sites of the carrier–hapten for subsequent linkages with the hapten–carrier (98) (Fig. 5). A number of coupling reagents, such as EDC (88) and glutaraldehyde (89), are available; the choice depends mainly on the free terminal groups available on the hapten being used.

2. Coupling Strategy

An antibody response to a hapten requires chemical attachment of the hapten to an immunogenic carrier. The choice of carrier proteins is based on certain criteria, such as solubility, molecular size, immunogenicity, commercial availability and cost (87). The most commonly used carrier proteins are KLH and BSA. Each has advantages and disadvantages: because of its large size, KLH tends to precipitate during coupling reactions, whereas BSA is soluble but less effective as an immunogen. In this study, both KLH and BSA were used for preparing conjugates for immunogen, and BSA or poly-L-lysine was used for preparing the screening conjugates. The molar ratio of carrier to hapten for the conjugation varied from 1:20 to 1:40, to achieve a good antibody response (90).

Most coupling procedures are based on the presence of free amino groups, carboxyl groups, and others, which are used to link haptens to protein carriers (99). As 5-S-CD possesses both amino and carboxyl groups, it can be coupled to protein carriers with coupling agents such as carbodiimide (*e.g.*, EDC, which acts on the carboxyl group) or glutaraldehyde (which acts on amino groups). EDC activates the carboxyl groups, mostly those on the hapten, to render them reactive towards amino groups on the carrier, but it also cross-reacts with amino groups

(88). Therefore, the hapten 5-S-CD was treated with citraconic anhydride before the coupling reactions, to block the amino groups of 5-S-CD in order to prevent the cross-reaction of EDC; this is termed citraconylation (92). Another bifunctional coupling reagent used in this study was glutaraldehyde, which forms a bridge between two amino groups of a hapten and a protein carrier (89). However, glutaraldehyde is a very strong cross-linker. As the hapten 5-S-CD possesses more than one free amino group, a limited amount of glutaraldehyde is required to minimize overcoupling; therefore, the concentration of glutaraldehyde used in this study was 0.05% or 0.1% (w/v), as recommended by Mueller's group (90).

On the other hand, the efficiency of the coupling experiments, also, is important in the preparation of immunogens. In the present study, 20% TCA was used to precipitate the 5-S-CD–protein conjugate in the mixture from pre- and post-coupling reactions, for comparison of the concentrations of free 5-S-CD left in those mixtures. As 5-S-CD is able to absorb UV light, this condition permitted determination of whether the hapten was coupled to the carrier by comparison of the UV absorbance values of the conjugate and of the protein carrier alone at the same protein concentration. If the UV absorbance of a conjugate increases, the hapten is believed to be coupled to the protein carrier. Poly-L-lysine cannot absorb UV light at 280 nm; therefore, using this as the carrier allowed an estimate of the efficiency of coupling experiments, by comparison of the UV absorbance values between the conjugate and the hapten 5-S-CD.

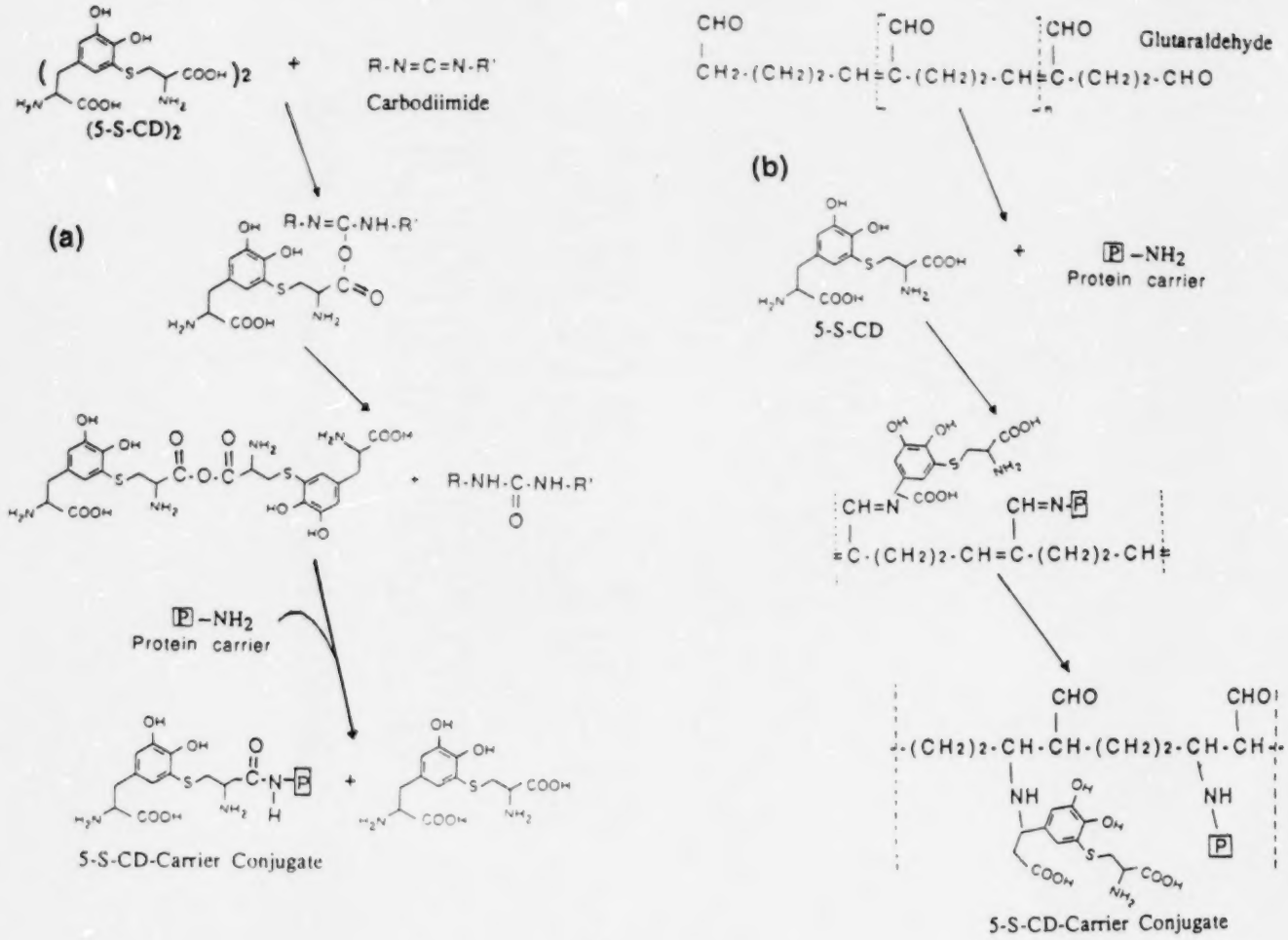


Figure 5
Postulated Mechanisms of Conjugations of 5-S-CD and the Protein Carrier Coupled with Carbodiimide (a) and Glutaraldehyde (b)

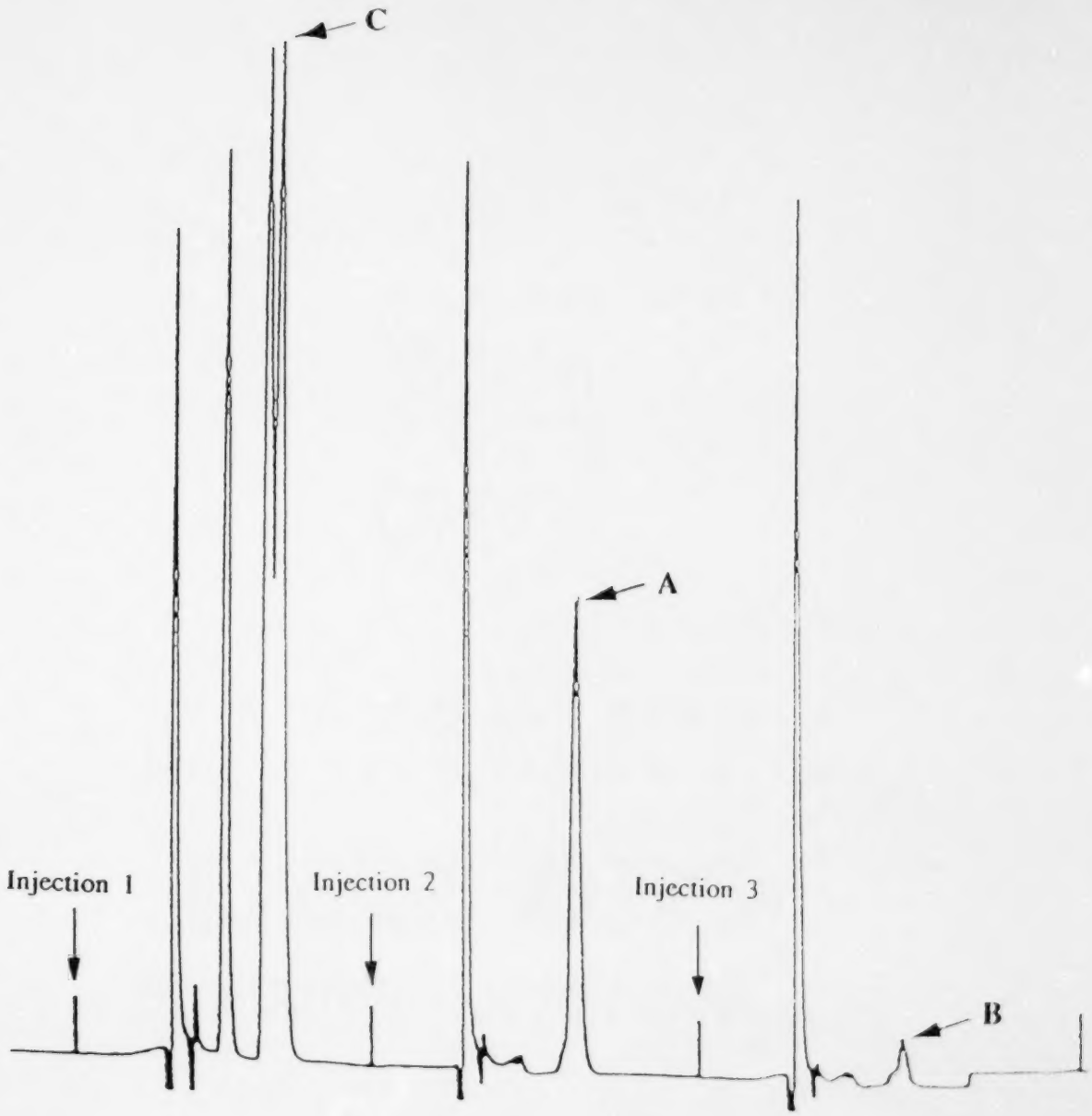


Figure 6
HPLC Chromatograms of Free 5-S-CD

Samples were injected separately, and the concentration of free 5-S-CD in each was calculated from the height of its 5-S-CD peak. A: Before coupling; B: After coupling; C: Control

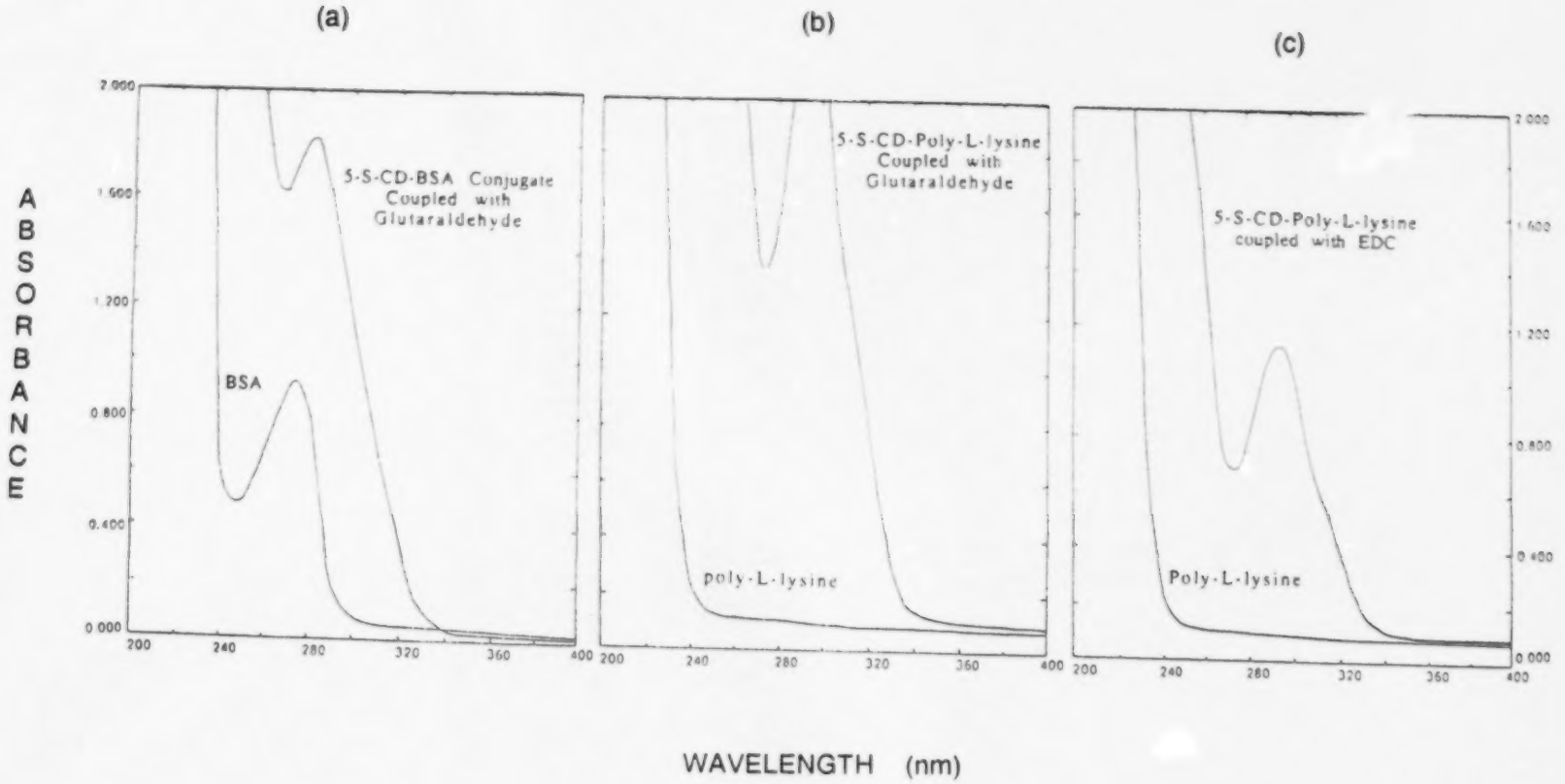


Figure 7
UV Absorbance Values of (a) 5-S-CD–BSA and (b) 5-S-CD–Poly-L-lysine Conjugates Coupled with Glutaraldehyde, and (c) 5-S-CD—Poly-L-lysine Conjugate Coupled with EDC

Using 0.01 M modified PBS as the background control, the UV absorbance was scanned at wavelengths from 200 to 400 nm by placing each sample (1 mL) in a spectrophotometer. (Scanning speed, 500 nm/min)

CHAPTER 4
IMMUNIZATION

A. INTRODUCTION

Antibody responses are the culmination of co-operation between antigen-presenting cells (APC) and helper T and B lymphocytes, all of which react to a foreign antigen (94). Therefore, the initial step for mounting an antibody response is immunization of the subject (*e.g.*, a mouse) with the desired immunogen (*e.g.*, 5-S-CD–carrier conjugate). Although the primary antibody response to an antigen is relatively weak, subsequent stimulation with the same immunogen can induce a strong secondary antibody response; this is due to the increased number of antigen-specific B cells formed during the primary response (100, 101). After a mouse has been immunized and boosted several times with an immunogen, serum samples are titrated periodically to monitor the degree of immunization.

In the present study, 10 mice were immunized with a 5-S-CD–protein conjugate which elicited antibody responses to both hapten and carrier; thus, the immunized serum contained a mixed population of antibodies (polyclonal antibodies) specific to at least two epitopes (5-S-CD and protein carrier). The antibody specific for 5-S-CD in sera from immunized mice was examined by immunoprecipitation on ELISA plates, using 5-S-CD linked to a different carrier to avoid potential screening artefacts (95). Also, cells from cultured melanoma cell lines were examined with immunostaining to test the reactivities of the sera from the immunized mice.

B. Materials and Methods

1. Mice

Ten 6- to 8-week-old female BALB/c mice were purchased from Jackson Laboratory, Bar Harbor, ME, USA.

2. Tumor Cell Lines

Cultured cells of 3 human melanoma cell lines were used. These were HMV-I (kindly supplied by Dr T Kasuga, Tokyo Medical & Dental School, Tokyo, Japan), SK-MEL 23 and SK-MEL 118 (kindly supplied by Dr A Houghton, Sloan Kettering Cancer Center, NY, USA). Two non-melanoma cell lines, HeLa cells (human cervical cancer) and PC-12 cells (rat pheochromocytoma) were purchased from the American Type Culture Collection (ATCC), Rockville, MD, USA.

3. Media and Reagents for Cell Culture

Minimal essential medium (MEM) for tumor-cell culture, fetal-calf serum (FCS), peroxidase-labeled goat anti-mouse IgG (Fc specific), NP-40, and diaminobenzidine (DAB) were purchased from Sigma Chemical Company (St Louis, MO, USA). Complete and incomplete Freund's adjuvants and antibiotics were purchased from Gibco Bethesda Research Laboratory (Burlington, Ont.).

4. Lysis of Tissue Culture Cells

The cell lines used to prepare cell extracts for determining cellular concentrations of 5-S-CD were 3 human melanoma cell lines (SK-MEL 23, SK-MEL 118 and HMV-I) and two non-melanoma cell lines (HeLa cells and PC 12 cells). Cultured cells of these cell lines were scraped

from the tissue culture flask with a scraper, then a cell suspension of each cell line (10^5 cells/10 mL of 0.01 M PBS) was transferred into a 15-mL tube and centrifuged at 300 *g* for 10 min. After the removal of supernatant fluids, each pellet was exposed to 0.5 mL of the extraction buffer [150 mM NaCl, 1% NP-40 (w/v) and 50 mM Tris, pH 8.0] for 30 min at 0°C with occasional shaking. Then, the cell lysates were spun at 10,000 *g* at 4°C for 30 min, and the supernatant fluids were saved. These cell extracts were then used to measure 5-S-CD concentrations in the cells with HPLC. This was carried out by Dr K Yamada in this laboratory.

5. Immunization

A 0.3-mL aliquot of emulsified suspension, consisting of equal volumes of 0.01 M modified PBS (pH 7.2) containing the 5-S-CD-protein conjugate and complete Freund's adjuvant, was injected into each mouse subcutaneously (sc). The amount of 5-S-CD injected was approximately 0.05 mg, according to the estimated coupling efficiency of each coupling experiment. Two weeks later, 0.2 mL of emulsified suspension of the same PBS, containing 0.05 mg of 5-S-CD and incomplete Freund's adjuvant, was injected into each mouse intraperitoneally (ip); this boosting step was repeated ip 5 to 6 times at 2-week intervals. To monitor the antibody titer, blood samples were collected from the tail veins of the immunized mice for titration analysis after the third boost. Three days before cell fusion, 0.1 mL of the same PBS containing 5-S-CD-protein conjugate was injected intravenously (iv) into the mouse with the highest titer of antibody; the concentration of 5-S-CD in the final iv injection was approximately 0.05 mg to 0.1 mg per mouse.

6. Screening

a. By ELISA

The binding assay on an ELISA plate for specific antibody is depicted in Figure 8a.

Using 96-well microtiter plates, each well was coated with 0.1 mL of $NaCO_3$ buffer (pH 9.6) containing 5-S-CD-BSA or 5-S-CD-poly-L-lysine (0.01 mg 5-S-CD/mL) or 1% KLH (w/v) and 1% BSA (w/v) or 1% poly-L-lysine (w/v) alone. The plates were incubated overnight at 4°C; then, washed three times with 0.01 M modified PBS (pH 7.2) and each well was filled with 0.01 M PBS containing 1% BSA (w/v) for blocking. The plates were left for 2 h at room temperature, washed three times with PBS, and then 0.1 mL of hybridoma supernatant fluid was added to each well. After incubation of the plates for 1 h at room temperature, followed by washing 5 times, 0.1 mL of peroxidase-labeled goat anti-mouse IgG (Fc specific) was added to each well. The plates were again left for 1 h at room temperature, then washed 5 times with PBS; 0.1 mL of peroxidase substrate solution was added to each well and the plates were re-incubated for 10 to 15 min. Absorbance was determined at 405 nm with the ELISA reader (Sit-LabInstruments, Salzburg, Austria).

b. By Immunostaining of Cultured Cells

Varying amounts of 5-S-CD were present in the 5 cell lines used in this study. Three human melanoma cell lines, HMV-I, SK-MEL 23 and SK-MEL 118, contained 0.166, 26.6 and 0.012 µg of 5-S-CD per 10^5 cells, respectively, and were used for immunostaining. HeLa cells (10^5 cells), the control for non-neural crest cells, revealed no significant amount of

5-S-CD, whereas PC-12 cells (10^5 cells), the control for non-melanocytic neural-crest cells, contained 0.166 μg of 5-S-CD.

Melanoma or non-melanoma cells grown in the tumor-cell growth medium [0.1 mL MEM, 5% FCS (v/v)] were plated into each well of 96-well plates (10^3 cells per well), and incubated for 48 to 72 h (until the cells became confluent) at 37°C with 5% CO_2 in air. After washing 5 times with 0.01 M PBS, pH 7.2, the cells were fixed with 95% alcohol (v/v) for 5 min. The plates were washed five times with PBS, incubated with blocking solution [1% BSA (w/v) in 0.01 M modified PBS] for 1 h at room temperature, then washed with PBS again, and 0.1 mL of antibody supernatant fluid was added to each well. After 1 h at room temperature, and re-washing, the plates were incubated with goat anti-mouse IgG (Fc specific) labeled with horseradish peroxidase (0.1 mL/well) for 1 h and then washed 5 times with PBS. Diaminobenzidine (DAB) solution (0.1 mL) was added into each well, and the plate was incubated for 3 to 5 min. The reaction was stopped by immersion of the plates in tapwater. Results were evaluated with light microscopy.

C. RESULTS

1. Antibody Titers in Immunized Mouse Serum

The titration curves of the antibodies against 5-S-CD–KLH conjugates in the serum of immunized mouse #3 after the third boost are shown in Figure 9. The immunogen was the conjugate of 5-S-CD and KLH, the screening antigen was the conjugate of 5-S-CD and BSA, and both were coupled with EDC; BSA or KLH alone was also coated as a screening antigen on the ELISA plates. Mouse serum obtained before immunization was incubated with the same screening antigens for the negative

control. In Figure 9, reactivity was directed against the 5-S-CD–BSA conjugate but not against BSA or KLH alone, indicating that only the reactivity against the hapten 5-S-CD was present in the immunized mouse serum. The serum from non-immunized mice (control) showed no reactivity with the conjugates, BSA and KLH.

The titration curves of the serum of mouse #5 immunized with the conjugate 5-S-CD–BSA after the fifth boost are shown in Figure 10. The immunogen was the conjugate of 5-S-CD and BSA coupled with glutaraldehyde. The antigen coated on the ELISA plates for the screening was the 5-S-CD–poly-L-lysine conjugate, coupled with EDC in the presence of citraconic anhydride. Poly-L-lysine and BSA alone (with or without glutaraldehyde treatment), also, were used as screening antigens. The serum from the same non-immunized mouse was used as the negative control for the screening. At least three different reactivities are evident in Figure 10: the first is directed against the 5-S-CD–poly-L-lysine conjugate coupled with EDC, and the second and third are against either BSA or poly-L-lysine treated with glutaraldehyde. Only the first reactivity (positive with the conjugate of 5-S-CD-poly-L-lysine but negative with poly-L-lysine or BSA alone) was specific for the hapten 5-S-CD. This anti-5-S-CD reactivity gave the highest value, as shown in Figure 10; the other two reactivities, which represented antibodies directed against the residues modified by coupling agents (*see* Discussion later in this chapter) were lower. The serum obtained before immunization (control) did not react positively with any of the screening antigens.

Figures 9 and 10 may indicate that anti-5-S-CD antibodies were present in the immunized mouse sera and that these were able to recognize the 5-S-CD–carrier conjugates even at high dilutions.

2. Immunostaining of Cultured Cells

The sera from immunized mice reacted strongly with melanoma cells of the SK-MEL 23 line; the immunostaining, which had a granular pattern, was located within the cytoplasm of the melanoma cells (*not shown*). The same sera did not react with the other four cell lines, and the sera from non-immunized mice showed no reactivity with any of the cell lines used in this study.

D. Discussion

1. Requirements of the Immunization Protocol

The many variations in immunization protocols necessitate considerations of various factors, including the characteristics of the animal (rabbit or mouse) and the candidate antigen (soluble or insoluble) (102). In general, if the antigen is water-soluble, an adjuvant is required to prevent rapid catabolism (103). In this study, Freund's complete adjuvant was used for the immunization and Freund's incomplete adjuvant for subsequent boosts, to prevent a possible hypersensitive reaction to bacterium in the complete adjuvant. For all injections, immunogen solutions and adjuvants were thoroughly emulsified; also, the amount of 5-S-CD injected each time did not exceed 0.1 mg, as repeated injections of large amounts of an antigen might have led to immunologic tolerance (104). In view of the carrier effect, 5-S-CD attached to the same carrier was used for both primary and

secondary immunization, to obtain a strong secondary antibody response. In addition, each mouse was bled before immunization, to provide the negative control in subsequent screening, and after the third boost the antibody titers were determined periodically for planning the subsequent cell fusion. Finally, because the number of antigen-specific B cells (plasmablasts) has been shown to correlate with a high frequency of antigen-specific hybridoma production (105), 0.05 to 0.1 mg of coupled 5-S-CD without adjuvant was injected iv into a mouse 3 days before fusion, to increase the plasmablast population at the time of fusion.

2. Problems in the Test Bleed

Two major problems were encountered during screening when hapten–carrier conjugates were used as immunogens or screening antigens.

The first problem stemmed from the immunogenicity of carrier molecules. Because the carrier proteins were able to elicit antibody responses themselves, there were at least two types of reactivity in the serum of an immunized mouse. The reactivity of interest was specifically against 5-S-CD, and another reactivity was specific for the carrier protein KLH. In order to distinguish between the specificities of these two types of reactivities, 5-S-CD was coupled to a different protein carrier, BSA, and the 5-S-CD–BSA conjugate was employed as the antigen for testing the sera of immunized mice. As shown in Figure 9, the highest level of reactivity was directed against only the 5-S-CD–KLH conjugate, not against KLH or BSA.

In Figure 9, the potential modifying effects of the bifunctional reagent EDC in the coupling reactions were not considered, as bifunctional reagents affect not only coupling efficiency but also an antigen's immunogenicity: they may become incorporated into the final conjugate or activate certain sites of carriers or haptens for subsequent coupling (92). Thus, it was necessary to consider a second possibility, that certain residues of haptens or carrier molecules had been modified by the coupling agent. This modification was not directly involved in the formation of hapten–carrier linkages. When the mice were immunized with hapten–carrier conjugates, such modified residues might have elicited the formation of antibodies, specific to the modified region, that were unable to recognize the untreated carrier or the hapten itself (90). Thus, the antibodies directed against the residues modified by coupling agents could react strongly not only with the same carrier treated with a coupling agent but also with unrelated carrier proteins treated with the same coupling agent (*see* Figure 10). In such a case, the presence of antibodies in anti-serum raised against a hapten–carrier conjugate should be examined, either with the non-conjugated hapten or with the hapten coupled to a different carrier with a different coupling reagent. As shown in Figure 10, such examination revealed at least three types of antibodies in the serum of the immunized mouse, two of them belonging to the same class as those antibodies specific for the coupling-agent-modified residue. The antibody that recognized only the conjugate of 5-S-CD–poly-L-lysine coupled with EDC was the only one specific to 5-S-CD, and thus, was the focus of further study.

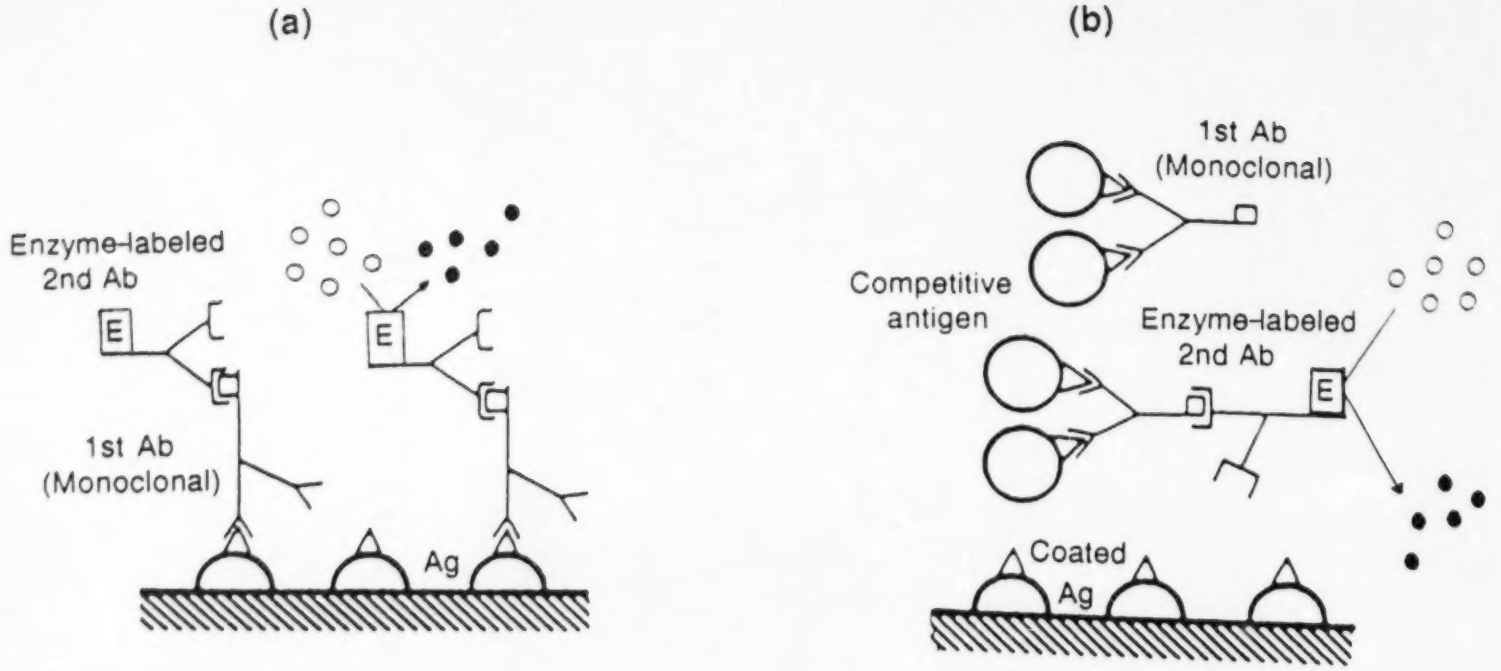


Figure 8
Solid-phase Binding Assays for Specific Antibody. a) ELISA
b) Competition ELISA. 0: substrate; ●: colored product

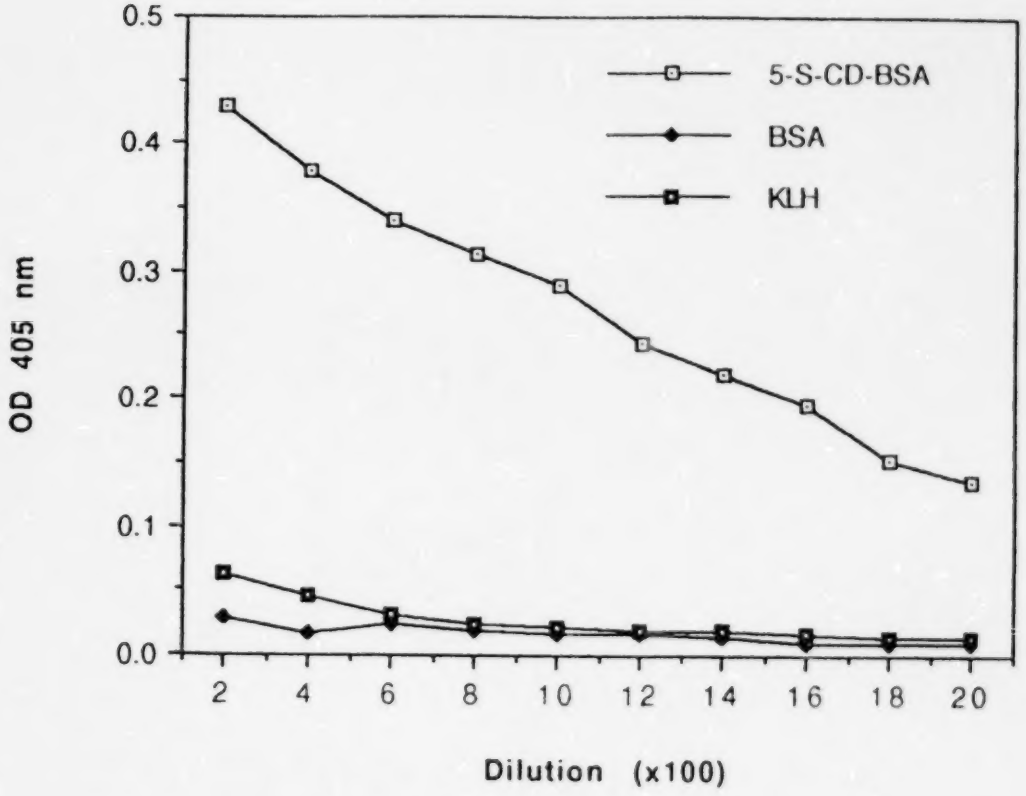


Figure 9
Titration Curves of Antibodies against 5-S-CD—KLH Conjugates Coupled with EDC in the Immunized Mouse Serum

After 1-h incubation of the antibodies with the antigens coated on ELISA plates, peroxidase-labeled goat anti-mouse antibody was added and incubation was continued for 1 h. Peroxidase substrate was then added and absorbance values were measured at 405 nm.

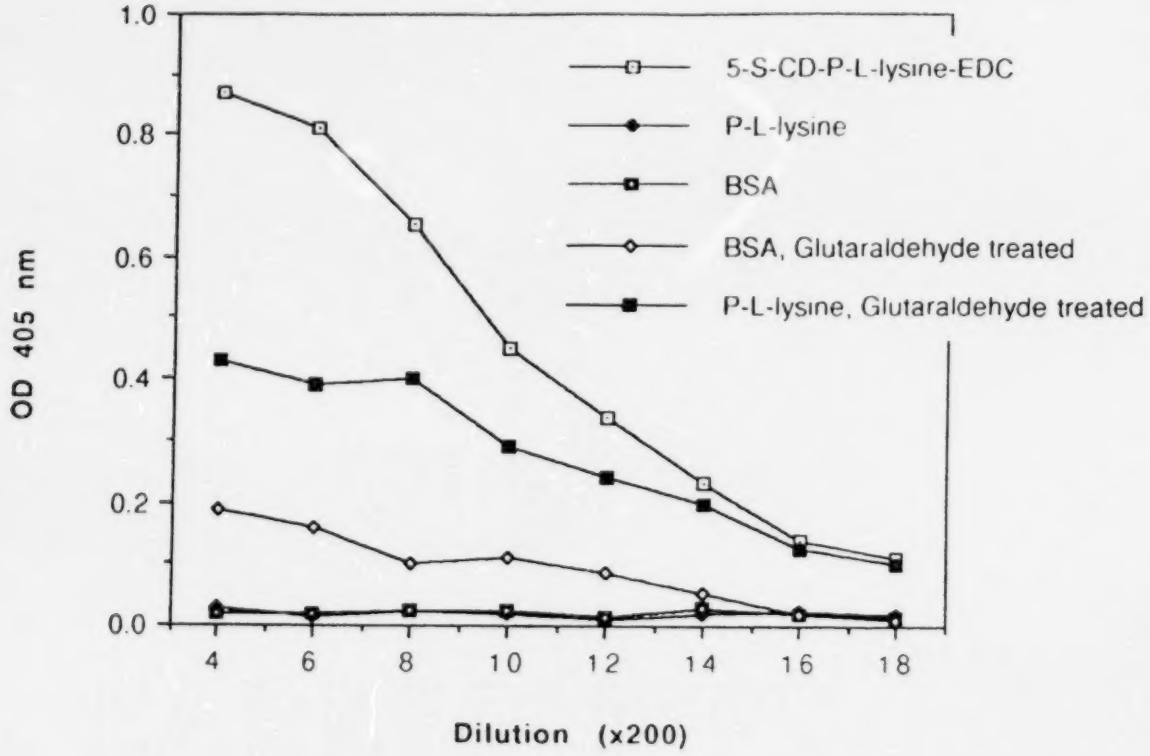


Figure 10
Titration Curves of Antibodies against 5-S-CD—BSA Conjugate Coupled with Glutaraldehyde in the Immunized Mouse Serum

See also the legend to Figure 9

CHAPTER 5
HYBRIDOMA DEVELOPMENT

A. INTRODUCTION

The development of homogeneous antibodies of defined specificity (*i.e.*, an antibody specifically against a particular antigen) was first achieved in 1975, by Kohler and Milstein (106), who established antibody-secreting hybridomas. In this technique, antibody-secreting cells are obtained from an immunized animal (*e.g.*, from the spleen of a mouse) and fused with myeloma cells (a type of B-lymphocyte tumor) with the fusogen polyethylene glycol. Because these myeloma cells have a defect in synthesizing hypoxanthine guanine phosphoribosyl transferase (HPRT), the key enzyme in the salvage pathway of purine synthesis, they cannot survive when the main pathway of purine synthesis is blocked by aminopterin, a folic acid analogue. This property makes it possible to select hybridomas (fused cells) from non-fused cells: the former can grow in medium containing hypoxanthine, aminopterin, and thymidine (HAT medium); whereas the unfused spleen cells survive only a few days and the unfused myeloma cells die from lack of HPRT (102). This scheme is illustrated in Figure 11. These hybrid cells (hybridomas) can be cultured *in vitro* indefinitely and can continue to produce antibodies with a desired specificity to a single epitope (106). An antibody produced by a hybridoma is designated a MoAb.

In this study, as BALB/c mice were used for the immunization, Sp2/0 myeloma cells derived from BALB/c mice (107) were employed for the development of hybridomas.

B. Materials and Methods

1. Mice

Ten 6- to 8-week-old female BALB/c mice were purchased from Jackson Laboratory, Bar Harbor, ME, USA.

2. Myeloma Cells

Mouse myeloma cell line Sp2/0 was purchased from American Type Culture Collection (ATCC), Rockville, MD, USA.

3. Media and Reagents for Cell Culture

The media used in this study were RPMI 1640 and the medium NCTC-109 for hybridoma culture, and minimal essential medium (MEM) for tumor cell culture. These media and fetal-calf serum (FCS) were purchased from Sigma Chemical Company, St Louis, MO, USA. Polyethylene glycol (PEG) 4000, was purchased from Merck, Darmstadt, Germany. The drugs for selecting hybridomas were hypoxanthine thymidine (HT) supplement (Gibco 320-1067) and aminopterin (Gibco 670-4070); they were purchased from Gibco Bethesda Research Lab, Burlington, Ont. Other chemicals, such as oxaloacetate, D-glucose, 2-mercaptoethanol (2-ME), sodium pyruvate, pristane, dimethylsulfoxide (DMSO), antibiotics (gentamycin and penicillin) and insulin were purchased from Sigma Chemical Company.

4. Cell Fusion

a. Preparation of Sp2/0 Myeloma Cells

Seven days before the fusion, Sp2/0 myeloma cells were grown in the medium for myeloma cell [10% heat-inactivated FCS (v/v), 1%

glutamine (v/v), 0.5% gentamycin (v/v), 0.2% 2-ME (v/v)]. On the day of fusion, the cells were harvested from petri dishes into the centrifuge tubes (50 mL). The cell suspension was spun for 10 min at 1000 rpm (300 *g*) and the pellets were resuspended in 50 mL of the washing medium [1% gentamycin (v/v), 0.5% 2-ME (v/v), in RPMI 1640]. The cells were counted with a hemocytometer.

b. Preparation of Macrophages

One day before cell fusion, 10 mL of autoclaved 11.6% sucrose solution was injected into the peritoneum of a mouse which had the same genetic background as the immunized mice. The belly of the mouse was massaged gently, and the solution was aspirated with a syringe (10 mL) fitted with a 23-G needle (to avoid sucking out tissues) and expelled into a 15-mL tube. The number of macrophages was counted with a hemocytometer, based on the size of cells under a light microscope (macrophages are larger than lymphocytes). The solution was centrifuged for 10 min at 1000 rpm (300 *g*), and the pellet was suspended in hybridoma medium [20% FCS-heat inactivated (v/v), 10% NCTC-109 (v/v), 1% HAT (v/v), 1% oxaloacetate (v/v), 0.5% 2-ME and 0.5% gentamycin, 1% sodium pyruvate (v/v); 2.25 g D-glucose and 400 IU insulin, in 500 mL RPMI 1640). Finally, 1 mL of macrophage suspension was plated into each well of 24-well plates at a density of $3X10^4$ cells per well, and the plates were placed in a humidified incubator at 37°C containing 7% CO_2 in air.

c. Preparation of Immunized Splenocytes

The splenocyte medium (5% FCS-heat inactivated, 1% gentamycin and 0.5% 2-ME, in 15-mL RPMI 1640) was added to each petri dish (usually 2 dishes). After induction of anesthesia with halothane, the immunized mouse was fixed on a styrofoam board with 4 needles and a small incision was made on its belly. Then the skin was torn to reveal the peritoneal wall, which was incised over the splenic area. The spleen was removed and placed in a dish containing splenocyte medium, and the mouse was killed by cardiac puncture while still anesthetized. The spleen was freed of fatty and connective tissue and transferred to another dish containing the same medium which was injected into the spleen repeatedly through 23-G and then 26-G needles fitted to a 10-mL syringe. The resulting splenocyte suspension was pipetted into a 50-mL tube and left standing for 5 to 10 min to precipitate out small pieces of floating tissue, which were removed. The suspension was transferred into another 50-mL tube and the cells were counted with Turk's solution (which stains only lymphocytes).

d. Fusion

The Sp2/0 cells and spleen cells were washed 4 times with washing medium (FCS-free) and centrifuged at 1100 rpm (310 *g*) for 10 min. After the final wash, the two cell types were mixed, in a ratio of 5:1 (spleen:myeloma cells), and the mixture was centrifuged at 1100 rpm (310 *g*) for 10 min. All of the supernatant fluid was pipetted off. The tube containing the pellet was placed in a water-bath at 37°C, and 0.5 to 0.8 mL PEG 4000 (2 mg PEG, 2 mL double-distilled H_2O and 0.2 mL DMSO), warmed to 37°C, was added over 1 min with gentle shaking. The

tube was stirred gently for another 90 sec, and 10 mL of warmed washing medium was added slowly over 5 to 8 min with gentle shaking (drop by drop for the first 3 mL and then by slow stream). The tube was replaced in the waterbath for 20 min; 10 mL of warmed washing medium was added, and the mixture was centrifuged at 900 to 1000 rpm (300 *g*) for 10 min. The final pellet was resuspended in hybridoma medium at a final concentration of 10^7 spleen cells per mL; 1 mL of the suspension was plated into each well in 24-well plates which were incubated in a humidified incubator at 37°C with 7% CO_2 in air.

5. Subcloning Hybridoma Cells (Limiting Dilution)

The hybridoma medium (0.1 mL) containing feeder cells ($3X10^4$ macrophages per mL) was added into each well of a 96-well plate, which was incubated overnight in a humidified incubator at 37°C with 7% CO_2. Initially, the hybridoma cell suspension (0.1 mL/well) was transferred to the top well on the left hand of the 96-well plate and mixed by pipetting. One-in-two serial dilutions were carried out down the left-hand row (row A) of the plate (8 wells, 7 dilution steps), and 1-in-2 dilutions were carried out across the plate which was then placed in the humidified incubator. The supernatant fluid of the hybridoma cells was screened by ELISA after 7 to 10 days' incubation.

6. Growth and Storage of Hybridoma Cells

a. Cell Storage

One day before freezing, the healthy and rapidly dividing cells were split into fresh medium at a 1 in 10 dilution. On the day of freezing, the

cells were transferred to centrifuge tubes and spun at 1000 rpm (300 *g*) for 5 min at room temperature. The supernatant fluid was removed as completely as possible and the cell pellet was gently resuspended in chilled freezing medium [a. 50% RPMI, 40% FCS, 10% DMSO, v/v; b. 90% FCS, 10% DMSO, v/v]; final cell concentration was approximately $5X10^6$ to $5X10^7$ cells/mL. The DMSO solution at a concentration lower than 5% did not permit a good recovery of most cell lines. Cell suspensions, in 1-mL aliquots, were transferred to chilled freezing vials on ice. The vials were sealed tightly and placed in a freezing rack, which was stored at -20°C for 20 to 30 min and then at -70°C overnight. It was found best not to freeze too many vials at the same time, as prolonged exposure to DMSO was toxic to the cells. Finally, the vials were transferred to liquid nitrogen.

b. Cell Recovery after Storage in Liquid Nitrogen

The frozen vials were removed from the liquid nitrogen and transferred to a water bath at 37°C. When the cells were almost thawed (*i.e.*, only a small chunk of ice), the vials were moved to a tissue culture hood. The outside of the vial was wiped with 70% ethanol, the lid was removed, and the cell suspension was transferred to a 15-mL centrifuge tube. Fresh medium (10 mL) with 1-mL FCS was added very slowly into the centrifuge tube, which was then spun gently at 1000 rpm (300 *g*) for 5 min. The supernatant fluid was removed and the pellet was resuspended in 10 mL of fresh medium with 1-mL FCS. Finally, the cell suspension was transferred to tissue culture flasks and placed in a humidified 7% CO_2 incubator at 37°C.

7. Expanding the Hybridoma *in Vivo*

Ten to 14 days before the injection of hybridoma cells, BALB/c mice at least 12 weeks old, with the same genetic background as the hybridomas, were injected intraperitoneally (ip) with 0.3 mL of pristane solution (2,6,10,14-tetramethyldecanoic acid, 97% purity). This solution is an irritant and stimulates the mice to secrete nutrients and recruit monocytes and lymphocytes into their peritoneum; this creates a good environment for the growth of hybridoma cells. One day before they were to be injected with hybridoma cells, the mice were irradiated with 600 rad for 5 min. Prior to injection, the rapidly growing hybridoma cells were centrifuged and washed twice with 0.1 M PBS, pH 7.2. Not more than 0.3 mL of PBS solution containing $5X10^5$ to $5X10^6$ hybridoma cells was injected ip into each animal. Ascites began to build up within 15 days; when the mouse's abdomen was noticeably large, but before the animal had difficulty moving, as much fluid as possible was withdrawn gently with an 18-G needle attached to a 10-mL syringe. Each mouse could yield as much as 8 to 9 mL of ascites. The ascites were incubated at 37°C for 1 h, then at 4°C overnight, and finally spun at 5000 rpm (3000 *g*) for 20 min. An oily layer was removed and discarded and the supernatant fluid was removed from the cell pellet and saved.

C. Results

1. Conjugate of 5-S-CD–KLH as Immunogen

When the titer of the antibody exceeded 1:10,000 on ELISA, fusion was scheduled. Three weeks after the final boost and 3 days before

fusion, the mouse with the highest titer of the antibody specific to the conjugate and which reacted positively with cultured SK-MEL 23 cells was injected iv with the coupled 5-S-CD (about 0.05 to 0.1 mg) without adjuvant. This resulted in a large number of rapidly dividing plasmablasts at the time of fusion. Seven days after the fusion, each hybridoma clone was transferred into a well of 24-well plates with the feeder cells and 0.5 mL of hybridoma medium, and incubated for 24 to 36 h at 37°C with 7% CO_2. The ELISA was used for initial screening of the supernatant fluid of each hybridoma against the conjugate of 5-S-CD and BSA coupled by EDC; the fluid of clones that were positive were tested against BSA and KLH alone coated on the ELISA plates.

Four hybridoma clones (1B2, 3C5, 4D4 and 6D4) were selected for their positive reactivities with the conjugate of 5-S-CD-BSA and their negative reactivities with BSA or KLH alone. Subsequently, the supernatant fluids of these four hybridomas were used to stain the sections of paraffin-embedded pathologic tissues, SK-MEL 23 melanoma cells with a high level of 5-S-CD and HeLa cervical tumor cells with no 5-S-CD. Only the supernatant fluid of hybridoma 6D4, designated as MoAb 6D4, reacted positively on the tissue sections and with cultured SK-MEL 23 cells; this hybridoma was therefore selected for further study, and subcloned by the limiting dilution method. During the subcloning, only the wells with one clone were screened by the ELISA; wells with high optical density (OD) readings were selected for cloning, which was repeated two more times. The selected hybridoma not only recognized the conjugate of 5-S-CD via both ELISA and immunostaining of cultured cells but also reacted positively with sections of formalin-fixed and paraffin-embedded melanoma tissue.

2. Conjugate of 5-S-CD–BSA as Immunogen

One day before the fusion, 0.05 to 0.1 mg of conjugated 5-S-CD was injected iv to stimulate the multiplication of the antibody-producing plasmablasts. The next day, fusion was carried out as described. After 1 week in a humidified incubator at 37°C with 7% CO_2, hybridoma clones appeared at the bottom of wells of 24-well culture plates. Each clone was transferred individually into a well with macrophages and incubated for a further 24 to 48 h (dependent upon the cell density) in a humidified 37°C incubator with 7% CO_2. First, ELISA was used to screen the supernatant fluid of each hybridoma, using the 5-S-CD–poly-L-lysine conjugate coupled with EDC, with citraconic anhydride present. Subsequently, the supernatant fluids of clones that were positive were screened against BSA and poly-L-lysine alone on the ELISA plates.

Twenty-two clones reacted positively with the conjugate of 5-S-CD and poly-L-lysine coupled by EDC with citraconic anhydride present. All 22 hybridomas reacted negatively on subsequent testing with BSA or poly-L-lysine, with or without glutaraldehyde treatment.

The 22 original clones were then frozen in liquid nitrogen for storage, and the 10 with high OD reading were subcloned twice by the limiting dilution method (*see* Materials and Methods above). The supernatant fluids from the 10 hybridomas reacted positively with cultured SK-MEL 23 melanoma cells but were negative against HeLa cells (data not shown). Finally, the isotypes of the antibodies secreted from these hybridomas were determined with the isotyping kit and ELISA (*see* Materials and Methods in Chapter 6).

D. DISCUSSION

1. Fusogen Polyethylene Glycol (PEG)

The mechanism of fusion between cells is complex, involving: (a) cell agglutination; (b) membrane fusion; and (c) cell swelling, and the ideal environmental conditions for these three processes often vary (108). Theoretically, fusion between a myeloma cell and an antibody-secreting cell can be achieved with any fusogen (109); the commonest fusogen now in use for the hybridoma technique is polyethylene glycol (PEG). Pontecorvo (110) demonstrated the use of PEG with mammalian cells, fusing the plasma membrane of two adjacent cells and thus forming a single cell with two or more nuclei, termed a heterokaryon (111). However, the exact fusion mechanism of PEG is still not well understood.

PEG, especially with lower molecular weights, is toxic to cells, the degree of toxicity varying with each cell type (112). PEG of high molecular weights is, however, viscous and difficult to work with (112); to date, most successful fusions have been achieved with PEG of molecular weights between 600 and 6000 Da (110, 112). In this study, PEG 4000 was used for cell fusion.

The rate of PEG addition and dilution is critical for successful fusion (109). In this study, the PEG was added over 1.5 min and then diluted by the addition of warmed washing medium over 8 min. In addition, 10% DMSO was used in the fusion solution because it considerably enhances the fusion frequencies (113). The nature of this DMSO effect is not fully understood.

2. Feeder Cells

To grow hybridoma cells effectively at very low density requires growth factors in addition to those supplied by normal tissue-culture media. These additional factors can be supplied by feeder cells (114), the commonest of which are peritoneal macrophages and spleen cells (115). Such cells usually have a limited lifespan in which they can nourish the emerging hybridomas before they die. In this study, phagocytic cells such as macrophages were used as feeder cells, as they could perform the additional function of removing the debris of cells killed by the aminopterin treatment. One day before fusion, peritoneal macrophages were harvested from BALB/c mice and were plated into 24-well plates at a cell density of $3X10^4$ cells/mL, 1 mL/well. This gave time for the feeder cells to become established and for us to ensure there was no contamination (which would have jeopardized subsequent fusion).

3. Single-cell Cloning

After a positive supernatant fluid has been identified, subcloning of the hybridoma cells is necessary at an early stage (116), for several reasons. (a) Fused cells carry a tetraploid number of chromosomes and, therefore, are genetically unstable and susceptible to nonspecific loss of chromosomes. Some of the lost chromosomes could have been encoded for antibody production; their absence would preclude the production of antibodies by these cells. (b) Antibody-secreting clones may grow slower than clones that do not produce antibodies: if a mixed population of hybridomas is not cloned early, such slower-growing clones may be overgrown and eliminated by non-antibody-secreting clones. (c)

Prolonged culture of antibody-secreting clones can result in further chromosomal loss and possible conversion to non-antibody-secreting clones.

These three problems result in the retention of only non-antibody-producing hybridoma cells. Single-cell cloning ensures that cells producing the desired antibody are truly monoclonal and that the production of this antibody can be maintained. Therefore, the original positive wells were single-cell cloned by a limiting dilution method (117) soon after the preliminary screening, and this was repeated until at least 95% of the wells tested were positive. This ensured that the hybridomas were stable and truly monoclonal.

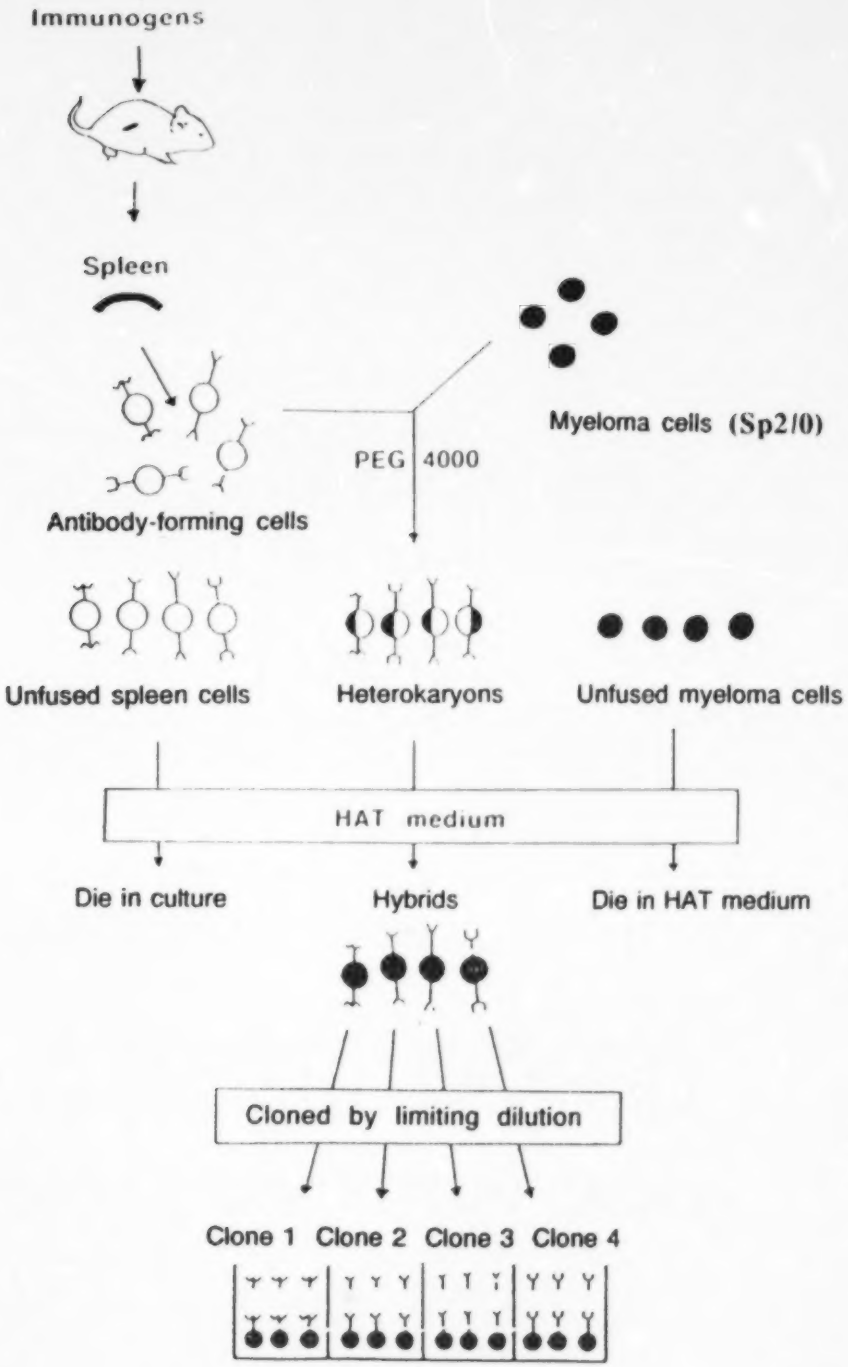


Figure 11
General Procedures of MoAb Development in Mice
(adapted from ref. 102)

CHAPTER 6
CHARACTERIZATION OF MoAb 6D4

A. INTRODUCTION

MoAb may be the most useful tool for detecting and isolating a particular molecule in a complex mixture and can provide information about the presence or location of a particular epitope (102). However, the complete characterization of a new MoAb is a long and complex process that varies widely with the intended use of the antibody.

In this study it was necessary to determine the reactivity of the new MoAb 6D4 against a group of different or similar antigens, to assess its specificity for antigens of similar structure to 5-S-CD and to examine its reactivity with precursors or intermediates of both eumelanogenesis and pheomelanogenesis within melanocytes.

In addition, the information of a MoAb's class and subclass is important in determining the strategy for future purification; for MoAb 6D4, these were determined after the hybridoma had been cloned three times. Various types of immunoassay were employed, such as the sub-isotyping assay, the inhibition ELISA (Fig. 8b), and both direct and indirect immunoperoxidase staining, to characterize the new MoAb and to detect and locate the antigen 5-S-CD within melanocytes.

B. MATERIALS AND METHODS

1. Chemicals (Reagents)

All reagents/chemicals were purchased from Sigma Chemical Company (St Louis, MO, USA). These included 2,2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid (ABTS), alanine, phenylalanine,

dopa, diaminobenzidine (DAB), Mouse-Typer Sub-isotyping Kit (goat anti-mouse subclass antibodies, peroxidase-labeled rabbit anti-goat antibody), ABC kit (normal horse serum, biotinylated goat anti-mouse antibody and avidin–biotin complex), and horseradish-peroxidase-labeled goat anti-mouse IgG (Fc specific). Eumelanin, pheomelanin and 5-S-CD were kindly supplied by Dr S Ito (Toyoake, Japan).

2. Sub-isotyping Assay

The conjugate of 5-S-CD–BSA or 5-S-CD–poly-L-lysine (approximately 0.01 mg/mL) in Na_2CO_3 buffer (0.1 mL) was coated in each well of 96-well microtiter plates which were then incubated at 4°C overnight. Any unbound antigens were removed by flooding with 0.01 M modified PBS (pH 7.2), and then the PBS was shaken off in a sink (flick-washed); this procedure was repeated 2 more times. To prevent nonspecific binding, the wells were filled with 0.2 mL blocking solution [1% BSA (w/v) in PBS] and incubated at room temperature for 3 h, then flick-washed 3 times with the PBS containing 0.05% Tween 20 (v/v). Supernatant fluid from individual hybridomas (undiluted, 0.1 mL) was added in duplicate to 6 wells of columns 2 to 11; column 1 was reserved for a second antibody blank and column 12 was for positive and negative controls (Fig. 12). The plate was covered and incubated for 1 h at room temperature. After removal of the hybridoma supernatant fluid, the plate was flick-washed three times with the PBS–Tween solution and the wells in rows A to H were filled with rabbit anti-mouse antibodies (0.1 mL/well). The plates were incubated for 1 h at room temperature, the rabbit antibodies were removed; the plate was flick-washed 5 times with the PBS–Tween solution, and wells in column 2 to

12 were filled with goat anti-rabbit–horseradish-peroxidase conjugate (1000-fold dilution, 0.1 mL/well). Then the plates were re-incubated for 1 h at room temperature, during which time the peroxidase-substrate solution was prepared. At 1 h, any unreacted conjugate solution was removed by flick-washing five times with the PBS–Tween solution; the plate was inverted and tapped against paper towels to remove excess washing solution and 0.1 mL of the peroxidase-substrate solution was added to each well. After 20 to 30 min incubation at room temperature, the antibody's class and subclass were assessed by absorbance, determined at 405 nm with the ELISA reader (Sit-Lab Instruments, Salzburg, Austria).

3. Indirect Immunoperoxidase Staining

Cellular antigen distribution was determined by immunostaining of formalin-fixed, paraffin-embedded sections with the new antibody, using the avidin–biotin complex (ABC). After deparaffinization (following the method decribed in ref. 68), the sections were treated with methanolic hydrogen peroxidase [200 mL methanol with 2 mL of 0.3% H_2O_2 (v/v)] to block the endogenous peroxidase activity (68), then washed with PBS and blocked with normal horse serum. The MoAb from undiluted culture supernatant fluid, with non-immunized mouse serum (the negative control) and 0.01 M modified PBS at pH 7.2 (the reagent control) were applied and left overnight at 4°C. After washing 3 times with PBS, the sections were incubated with biotinylated goat anti-mouse antibody for 30 min and with the avidin–biotin complex for a further 30 min, and then with the substrate solution including 0.025% DAB (w/v) and 0.01% hydrogen peroxide (v/v) for 3 min. Finally, the sections were

counterstained with 1% methyl green (w/v) to differentiate the reaction product from melanin pigments (68).

4. Inhibition ELISA

An inhibition ELISA was developed to evaluate the potential cross-reactivity of the MoAb with pheomelanin and other compounds structurally similar to 5-S-CD (Fig. 13). Briefly, the wells of 96-well microtiter plates were coated with 0.1 mL of carbonate buffer containing 5-S-CD-BSA (approximately 1 μg of 5-S-CD per well) and incubated overnight at 4°C, washed with PBS, filled with 1% BSA (w/v) and re-incubated for 3 h. The primary antibodies (0.1 mL of supernatant fluid) were first incubated with PBS (0.1 mL) containing a known amount of inhibitors for 30 min in 1.5-mL polypropylene tubes. After removal of the blocking buffer from the wells, the above mixtures (primary antibody and inhibitors) were added to the wells in triplicate and left for 1 h at room temperature. The plates were washed 3 times with PBS, and then 0.1 mL of horseradish-peroxidase-labeled goat anti-mouse IgG (Fc specific) was added to each well. The plates were left for 1 h at room temperature, then washed 5 times with PBS; 0.1 mL of substrate solution (0.05 M sodium citrate, 0.5 M hydrogen peroxide and ABTS) was added to each well and left for 10 min. Finally, absorbance values were determined at 405 nm with the ELISA reader.

C. Results

1. Specificity of MoAb 6D4

By using synthetic pheomelanin, eumelanin, and other compounds structurally similar to 5-S-CD, the specificity of the MoAb 6D4 (IgG_1, κ)

was evaluated with the competitive ELISA. The chemical structures of all inhibitors and the concentration required for 50% inhibition of each are shown in Table 3.

The binding ability of MoAb 6D4 was inhibited to varying degrees by the melanin precursors and their related compounds, as shown in Figure 14 and Table 4; its binding to the coated antigen 5-S-CD-BSA conjugate decreased with increased concentrations of the inhibitors, 5-S-CD, pheomelanin, dopa and phenylalanine. The results of this assay indicated that MoAb 6D4 strongly recognized 5-S-CD and pheomelanin but had only minimal reactivity with eumelanin. Alanine did not block the binding ability of MoAb 6D4 to the 5-S-CD-BSA conjugate; phenylalanine showed weak inhibition, and dopa significantly inhibited this MoAb's binding capability. The amounts of 5-S-CD, pheomelanin, dopa and phenylalanine causing 50% inhibition of antibody binding to the coated conjugate were 65, 95, 180 and 2500 ng/mL, respectively. The minimal amount of non-conjugated 5-S-CD detected by MoAb 6D4 in this inhibition ELISA was 5 ng per mL.

2. Reactivities of MoAb 6D4 with Normal and Diseased Tissues

The reactivity of MoAb 6D4 on formalin-fixed, paraffin-embedded sections of malignant melanoma, melanocytic nevi, non-melanocytic tumors, and normal foreskin from newborns is summarized in Table 5. There was no reactivity in any of 23 melanoma tissues, including nodular melanoma, lentigo maligna melanoma and lentigo maligna, but positive reactivity was seen in three of eight specimens of superficial spreading melanoma (SSM). This indicates that the antigen(s) recognized by this new MoAb was located in only certain types of melanocytic

tumors. Immunostaining of the sections from SSM is shown in Figure 15. A low-power view (Fig. 15-Aa) reveals positive staining of tumor-cell nests with MoAb 6D4, the pattern and degree of which varied among individual tumor cells in the nests (so-called heterogeneous reactivity). Below the tumor-cell nests, there were inflammatory cells, such as lymphocytes and macrophages, which showed negative reactivity with the MoAb. A high-power view (Fig. 15-Ab) indicates that the positive staining was located in the cytoplasm of tumor cells; the surrounding keratinocytes and other structures (*e.g.*, blood and lymphatic vessels, sebaceous glands and arrector muscles) did not show any positive reactions. The control specimen (melanoma tissue incubated with non-immunized mouse serum) remained unstained (Fig. 15B).

MoAb 6D4 had no reactivity with melanocytic nevi, including dysplastic melanocytic nevi (DMN), and no cross-reactivities with non-melanocytic tumors of skin or with tumors of other tissues derived from the neural crest such as neurofibroma and schwannoma. There was also no positive reactivity with melanocytes and keratinocytes in normal skin. In addition, immunostaining of melanoma cell line SK-MEL 23 also indicated that the antigen was expressed in the cytoplasm of alcohol-fixed human melanoma cells (not shown).

D. Discussion

1. Specificity and Reactivity of MoAb 6D4

The results obtained in these experiments indicated several unique features of MoAb 6D4, which was directed against the pheomelanin precursor 5-S-CD. First, the antigenic epitope recognized by MoAb 6D4 was located in the cytoplasm of SK-MEL 23 melanoma cells (not shown).

On competitive ELISA, MoAb 6D4 reacted strongly with 5-S-CD, and to a much lesser extent with analogs such as pheomelanin and dopa (Fig. 14). As no cross-reactivity with eumelanin or alanine was detected, it is assumed that the antigenic epitope recognized by this MoAb consists of a phenolic ring in conjunction with a sulfur atom or with either an amino or carboxylic acid group or both. The cross-reactivity of 6D4 MoAb with 5-S-CD analogs was not unexpected, in view of the high degree of the similarity in chemical structure between 5-S-CD and its related compounds (Fig. 13) and the potential modification of immunogenicity of haptens by the coupling reagent (*see* Discussion in Chapter 3).

The results of the indirect immuno-peroxidase assay indicated that the MoAb 6D4 was capable of recognizing the antigen(s) contained in several formalin-fixed and paraffin-embedded tissue sections. The antibody exhibited strong reactivity with SSM, but minimal reactivity with DMN or the other types of melanomas. These results were different from expected, because previous studies in this laboratory indicated a high level of pheomelanic components in DMN (47, 118). These unexpected results may be due partly to tissue fixation. As 5-S-CD is easily solubilized, significant amounts of its free form may have been dissolved out during tissue processing. In the case of specimens with a relatively lower level of 5-S-CD or pheomelanin, as in DMN, such losses could result in lack of reactivity with the antibody, whereas losses from specimens containing a higher concentration of these compounds (*e.g.*, SSM) would cause a negligible difference. In addition, several previous studies have indicated that pheomelanin and its intermediates, such as 5-S-CD, are present as a non-particulate, soluble form in melanocytes

(11, 57), a form that would be readily lost from tissues during processing. Positive reactivity of this MoAb 6D4 with pathologic tissues in cases of melanocytic disease might be greater if frozen tissue sections were used, a method less likely to damage the components of tissues and free the 5-S-CD. Unfortunately, it was difficult to obtain frozen tissues to examine their reactivity with this MoAb.

The type of melanin synthesized correlates with the morphology of melanosomes produced by normal melanocytes, a relationship that can be changed in transformed melanocytes—probably as a result of alterations in the structural matrix protein (119). For example, melanosomes in SSM are partly melanized spherical granules with a much higher level of pheomelanin components, similar to those in DMN lesions. On the other hand, melanosomes in LMM, like those in normal epidermal melanocytes, are highly melanized ellipsoidal granules rich in eumelanin components. This observation was supported by previous studies, in which different amounts of 5-S-CD/pheomelanin were found in biopsy specimens from patients with benign and malignant melanocytic diseases (*e.g.*, DMN and SSM), and in the plasma and urine of patients with various subtypes of melanoma with or without metastasis (93, 118, 120). Thus, the results of this study directly or indirectly indicate that not all melanocytic tumors, whether malignant or benign, synthesize pheomelanin; or, alternatively, that they synthesize different amounts of pheomelanin in terms of 5-S-CD, in that a large percentage of this pheomelanin precursor was present in a soluble form within the cells.

2. Potential Clinical Application of MoAb 6D4

The synthesis of melanin pigments is greatly increased in malignant melanoma. The metabolites of these melanin pigments are released into the extracellular spaces and thence into the circulation and finally excreted into the urine (121). As the excreted 5-S-CD is mainly derived from melanocytes, one may expect a correlation between the increased level of 5-S-CD in the plasma or urine and the ability of melanocytes to synthesize pheomelanin. Agrup *et al.* (122-124) developed a method to quantify 5-S-CD in the plasma and urine with high-performance liquid chromatography (HPLC). Since then, the concentration of 5-S-CD in urine and plasma has been used as a biochemical marker of the progression of melanoma and development of metastases, and even for evaluating the effect of chemotherapy (125). These reports are summarized in Table 6.

Data from these and other studies indicate that the quantification of 5-S-CD in urine or plasma is a reliable laboratory method, not only for early detection of melanoma metastases, but also as an indicator of the metabolism of the pheomelanin pigment in healthy persons exposed to sunlight (83). The plasma level of 5-S-CD seems to be more accurate in the early detection of melanoma metastasis than its urinary excretion and to be the most sensitive marker of progression of the disease (82). Furthermore, a recent study of B16 murine melanoma indicated an excellent correlation between the plasma level of 5-S-CD and tumor weight but no such correlation with urinary excretion of this metabolite exists (126). Lately, in other studies, the eumelanin-related metabolites, the 6-*o*-sulfate derivative of 5,6-DHI and *o*-methylated derivatives of DHICA, were detected in plasma and urine from melanoma patients (127, 128), indicating that these eumelanin-related metabolites, also, can be

used as prognostic markers of malignant melanoma. Recent findings in this laboratory (93) have indicated that the combined measurement of these two markers (*i.e.*, 5-S-CD and eumelanin-related metabolites) is more reliable than either alone in detecting metastases at an early stage of malignant melanoma. However, the main drawback to the use of HPLC in quantifying these metabolites is the extensive preliminary purification of the body fluids or lesion tissues, which complicates and limits the use of this assay.

The many recently developed MoAbs directed against the molecules involved in melanogenesis (Table 2) have yielded more information on the biosynthesis of melanin pigments. Most recently, MoAb HMSA-5 was developed in this laboratory (129) against purified melanosomes isolated from cultured human melanocytes. Another MoAb, TA99, was reported to recognize PAA, the pigmentation-associated antigen (130). Both HMSA-5 and TA99 appear to recognize a common glycoprotein, gp75-PAA, which initially was described as a protein antigen immunoprecipitated with serum from a melanoma patient. This glycoprotein was detected in both normal and malignant melanocytes (131) and, importantly, was found to be encoded by the b-locus gene that encodes for tyrosinase-related proteins (TRP) (132). Recent studies have demonstrated that gp75–PAA possesses a measurable catalase activity, indicating that it may function as an enzyme controlling melanin synthesis (38). In addition, a melanoma-associated glycoprotein (MAG) recognized by several MoAbs, such as ME491 and LS62, has now been confirmed as the lysosomal protein CD 63 (133); this antigen was present in melanocytes from nevi and both primary and metastatic melanomas but not in normal melanocytes (134). Ultrastructural studies

have shown that the MAG/CD63 defined by ME491 is located in lysosomes and can be transported to the nucleus, where it is associated with a 55-kDa protein and two DNA sequences (135). However, the true function of this glycoprotein is unknown and the antibodies described above have not yielded information on melanin biosynthesis itself.

The MoAb 6D4 obtained in this study appears to be the only MoAb directed against 5-S-CD and pheomelanin at present. It has a high degree of sensitivity for 5-S-CD and, therefore, may be employed for detecting this in urine, plasma, and other types of tissue samples with immunoassays such as the inhibition ELISA and immunostaining. In this study, the lowest detectable amount of 5-S-CD by MoAb 6D4 in a competitive ELISA system was 5 ng per mL. Because this MoAb is able to react with SK-MEL 23 melanoma cells, it may be possible to determine the distribution of 5-S-CD or pheomelanin within melanocytes with the aid of immunoelectron microscopy. Thus, MoAb 6D4 may provide a new and convenient means not only to detect 5-S-CD in body fluids (*e.g.*, urine and plasma) but also to investigate the subcellular distribution of this pheomelanin precursor.

Unfortunately, MoAb 6D4 possesses a fairly broad specificity of antigen recognition and recognizes not only 5-S-CD but also several other compounds of similar structure, such as pheomelanin and dopa. This broad specificity detracts from its potential use in clinical diagnosis. However, by establishing other MoAbs recognizing 5-S-CD and incorporating them into a single test reagent, it might be possible to overcome the broad specificity of MoAb 6D4 and further improve the sensitivity and specificity of the tests.

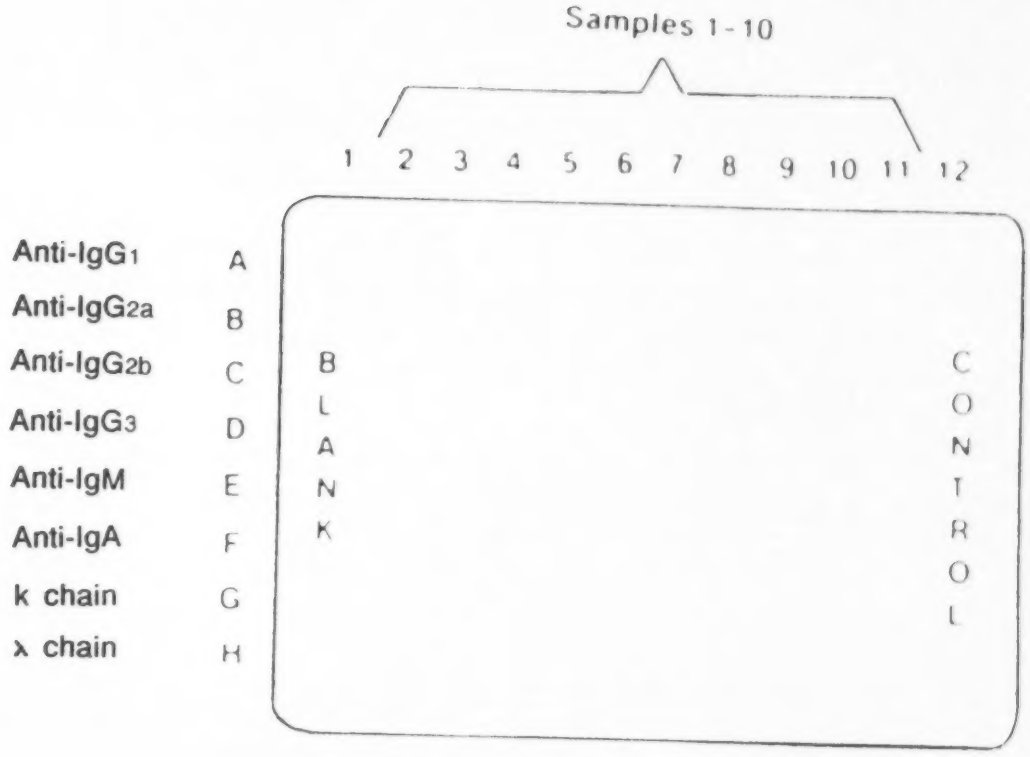


Figure 12
The Format for Sub-isotyping Assay on One Microtitration Plate

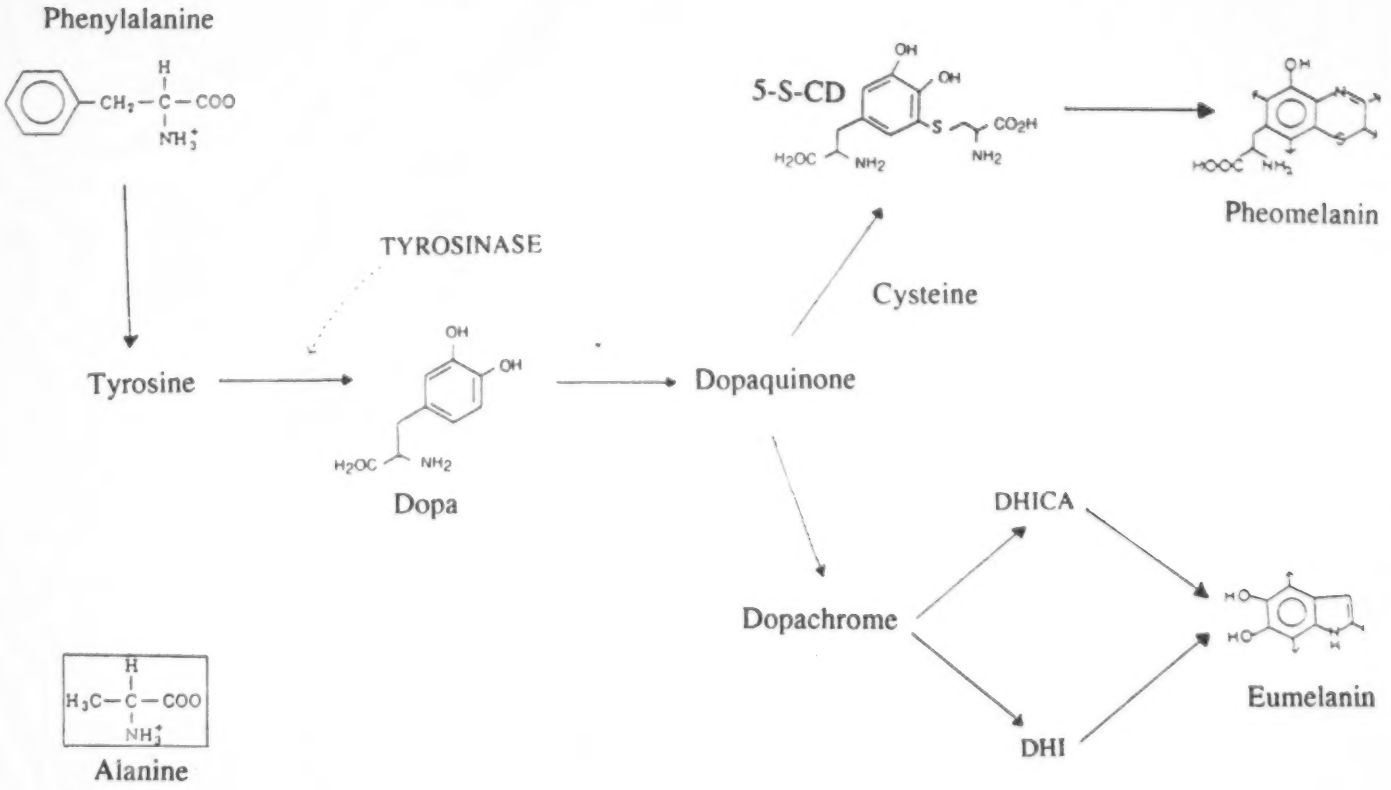


Figure 13
Role of 5-S-CD and Its Related Compounds in the Synthesis of Melanin

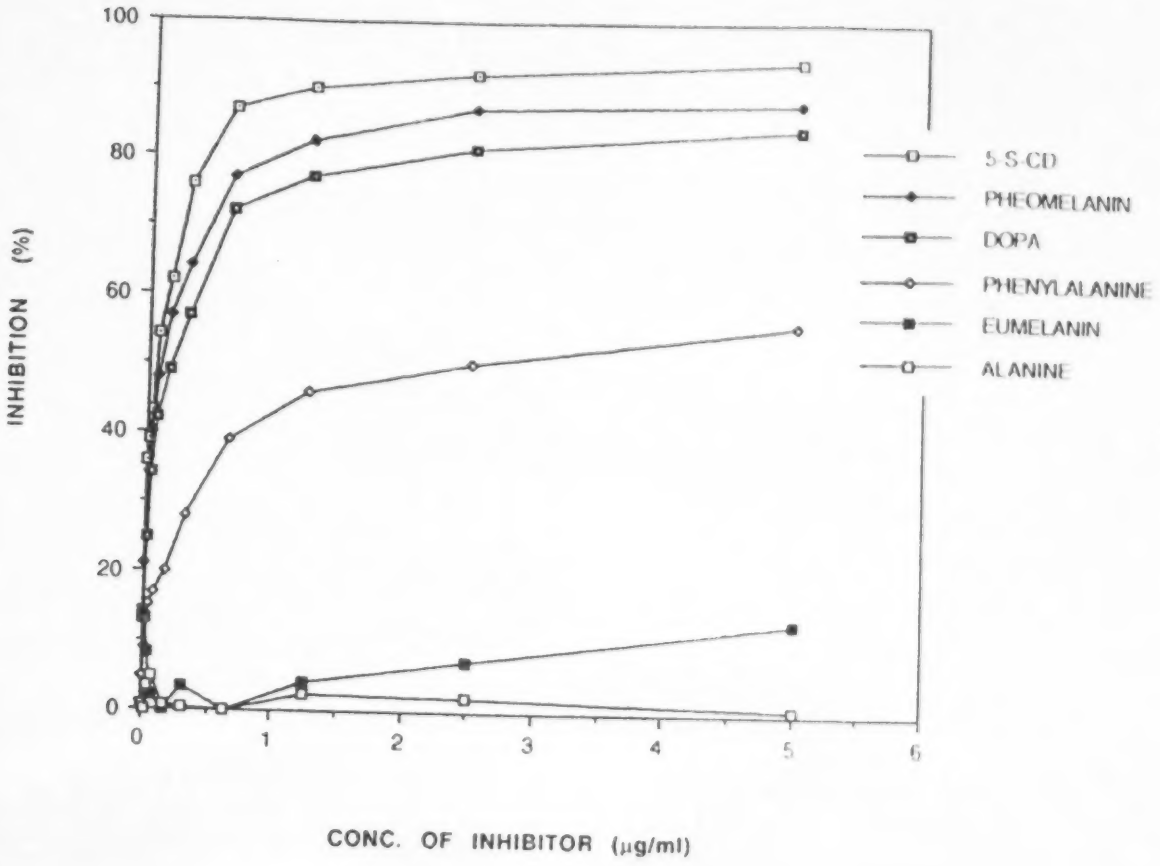
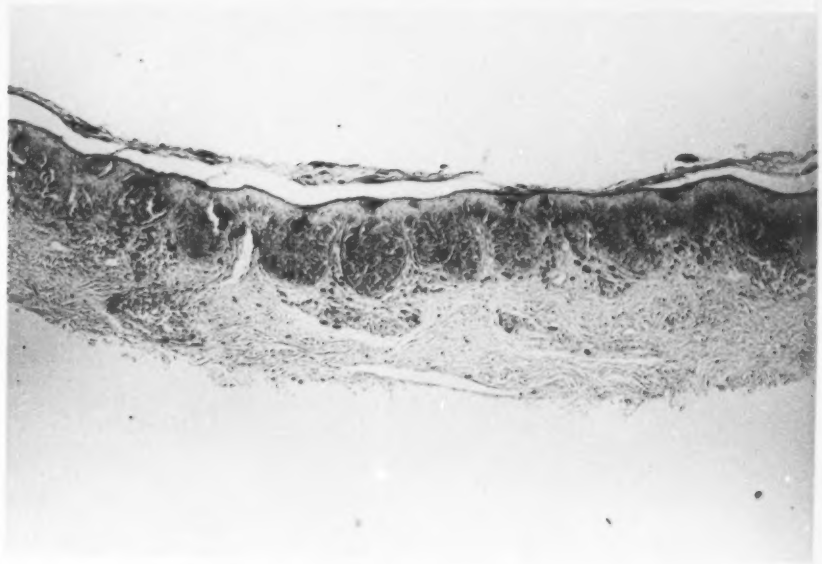


Figure 14
Inhibition of MoAb 6D4 by 5-S-CD and Its Related Compounds

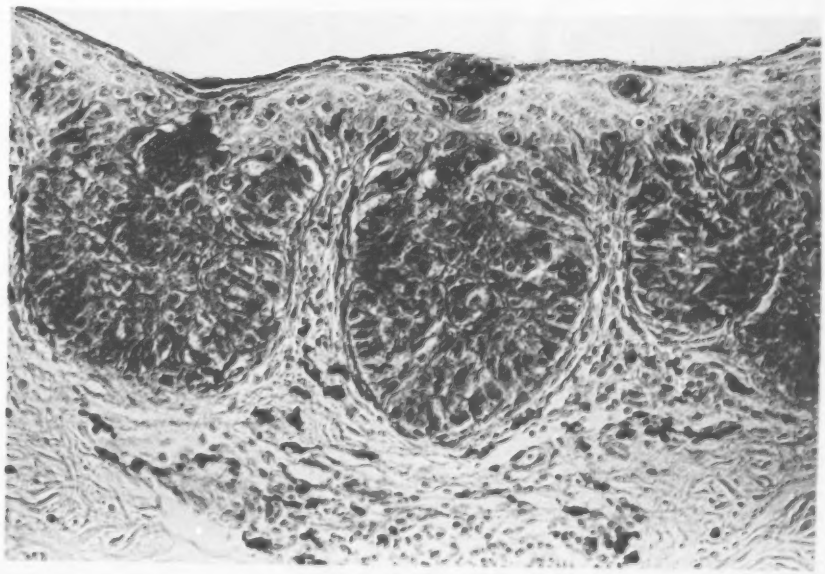
Before 2-h incubation with the conjugate of 5-S-CD-BSA coated on the wells of ELISA plates, MoAb 6D4 was incubated with synthetic 5-S-CD and its related compounds. Then the peroxidase-labeled 2nd antibody and the substrate of peroxidase were added separately into the wells. Finally, the absorbance values were measured at 405 nm and the binding inhibition values were calculated.

Figure 15
Avidin–Biotin Complex Staining of Formalin-fixed and Paraffin-embedded Sections of Superficial Spreading Melanoma

a)



b)



A: With MoAb 6D4. (a) Low-power View x 100. (b) High-power View x 1000 (oil)

2 of/de 2

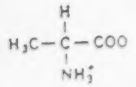
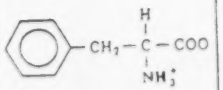
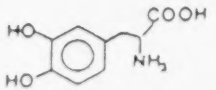
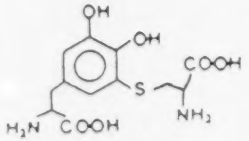
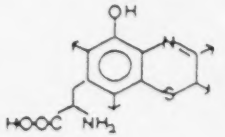
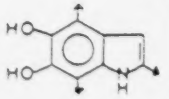
1.0
1.1
1.25
1.4
1.6
2.8
3.2
3.6
4.0
2.5
2.2
2.0
1.8

a)

b)

B. With Non-immunized Mouse Serum. (a) Low-power View x 300. (b) High-power View. x 1000 (oil)

Table 3 Values of 50% Binding Inhibition of MoAb 6D4 by 5-S-CD and Its Related Compounds

	Alanine	Phenylalanine	Dopa	5-S-CD	Pheomelanin	Eumelanin
						
50% Binding Inhibition (ng/ml) *	—	2500	180	65	95	—

*Before 2-h incubation with the conjugate of 5-S-CD-BSA, MoAb 6D4 was incubated with synthetic 5-S-CD and its related compounds. Then the peroxidase-labeled 2nd antibody and the substrate of peroxidase were added separately into the wells. Finally, the absorbance values were measured at 405 nm and the 50% inhibition values were calculated.

Table 4 Inhibition of MoAb 6D4 by 5-S-CD and Its Related Compounds

Conc. of Inhibitor (μg/mL)	% Inhibition*					
	5-S-CD	Pheomelanin	Dopa	Phenylalanine	Eumelalnin	Alanine
5	94±6.3%	88±4.2%	84±3.5%	65±4.3%	13±2.8%	0.8±7.4%
2.5	92±3.5%	87±0.7%	81±2.1%	60±4.3%	7.2±6.3%	4.1±6.6%
1.25	90±6.3%	82±2.1%	77±0.7%	56±2.6%	4.2±6.3%	2.4±3.3%
0.625	87±3.5%	77±4.2%	72±3.5%	49±5.2%	0	0
0.312	76±1.4%	64±6.3%	57±5.0%	39±4.3%	3.5±3.5%	0.2±4.9%
0.155	62±4.9%	57±3.5%	49±3.5%	28±0.8%	0	0.8±3.3%
0.078	54±4.2%	48±3.5%	42±4.2%	22±2.6%	2.1±2.8%	4.9±4.9%
0.039	43±3.5%	40±2.8%	34±4.2%	15±3.5%	8.4±3.5%	3.3±7.4%
0.019	39±4.2%	34±2.1%	25±3.5%	9±1.7%	13±2.8%	0
0.009	36±4.2%	21±1.4%	13±1.4%	5±4.3%	14±6.3%	0.8±3.3%
Control	0	0	0	0	0	0

See the legend to Figure 14

* n=3; Mean±Standard Deviation

Table 5 Reactivity of MoAb 6D4 with Normal and Pathologic Human Tissues

Type of Tumors/Tissues	Reactivity of MoAb 6D4 on Paraffin Sections
Melanocytic Skin Tumors	
Malignant Melanoma	
Superficial spreading melanoma	3/8
Lentigo maligna melanoma	0/6
Metastatic melanoma	0/6
Dysplastic Melanocytic nevi	0/6
Congenital Melanocytic nevi	0/5
Tumors Deriving From Other Than the Neural Crest	
Adenocarcinoma	
Breast	0/2
Lung	0/2
Stomach	0/1
Urinary bladder	0/1
Pancreas	0/2
Hepatoma	0/2
Non-melanocytic Tumors Deriving from the Neural Crest	
Neurofibroma	0/3
Neuroma	0/1
Schwannoma	0/3
Astrocytoma	0/3
Paraganglioma	0/2
Normal Skin from Newborn Foreskin	
Melanocyte	0/2
Keratinocyte	0/2
Fibroblast	0/2
Other dermal cells	0/2

Table 6 5-S-CD as a Biochemical Marker Used in Detecting Progression of Human and Murine Melanomas

Samples /Species	Specificity & Significance	Ref.
Urine; Human	In 1973 Agrup *et al.* used HPLC to determine main values of 5-S-CD in men and women. There was no significant difference in relation to age or body weight, or in relation to hair color except for white hair.	83
	Since then, highly increased excretion of 5-S-CD has been reported for many patients who have metastatic melanoma. The 5-S-CD value was more accurate when other markers (*e.g.*, dopa and dopamine) were taken into account.	81, 124
Plasma; Human	In 1979, Hansson *et al.* used HPLC to quantify 5-S-CD in plasma and found the level of 5-S-CD significantly greater in melanoma patients than in normal subjects. Moreover, the 5-S-CD values in plasma were more reliable than the values in urine in early diagnosis of melanoma metastases.	123
	In 1981, Aubert *et al.* measured the plasma level of 5-S-CD of melanoma patients before and after chemotherapy. The findings indicated that this concentration could be used to evaluate the effects of the therapy.	125
Tissue; Human	In 1989, Kanematsu & Morishima observed that the concentration of 5-S-CD in lesions could be used in the diagnosis of early stages of both nodular and superficial spreading melanoma.	136
Plasma & urine; Murine	In 1990, Wakamatsu & Ito, in studies of B16 murine melanoma, reported excellent correlation between plasma level of 5-S-CD and tumor weight but no significant correlation between the urinary excretion of 5-S-CD and tumor weight.	127

CHAPTER 7

CONCLUSION

In this study a murine anti-5-S-CD MoAb designated as 6D4 was developed and its immunobiologic properties were characterized. In general, this project encompassed three independent but related topics: (a) the preparation of immunogens, (b) the establishment of MoAb 6D4, and (c) characterization of this new antibody.

Previous studies (136-140) have indicated that 5-S-CD is a highly sensitive marker for increased synthesis of pheomelanin and reflects abnormal melanin synthesis as the result of the transformation of epidermal melanocytes. Increased urinary excretion of 5-S-CD is seen as 'melanuria' in cases of disseminated melanoma. The plasma level of 5-S-CD, also, is greatly increased in melanoma with or without metastasis (82, 128).

Recent studies (93, 120) in this laboratory have shown that the plasma level of 5-S-CD increases in parallel with the metastatic spread of malignant melanoma and that the concentrations of eumelanin-related metabolites increase with progression of the disease. The concentrations of eumelanin-related metabolites were found to be more reliable than those of 5-S-CD in the early detection of disseminated melanoma, as 5-S-CD can be released into the urine and blood of healthy individuals exposed to sunlight. Because 5-S-CD is the most sensitive known marker for the increased synthesis of pheomelanin, it is necessary to measure both types of melanin metabolites (*i.e.*, 5-S-CD and eumelanin-related metabolites) for accurate detection of occult

progression of melanoma. However, the techniques for identifying 5-S-CD in the plasma and urine are limited and complicated. At present, the commonest method relies on HPLC, which is very time-consuming (141).

The MoAb 6D4, which has high immunoreactivity with 5-S-CD, can be used to develop a radioimmunoassay (RIA) for the detection of 5-S-CD in the plasma and urine; this antibody detected 5-S-CD concentrations as low as 5 ng/mL in the inhibition ELISA. As most RIAs are cheaper and easier to perform than the HPLC assay, MoAb 6D4 may provide a reliable and less-costly means for measuring 5-S-CD in body fluid (*e.g.*, urine and plasma) and for detecting melanoma metastases at an early stage. Furthermore, the accuracy of this RIA could be increased by combining MoAb 6D4 with other antibodies, such as those against eumelanin-related metabolites. However, no other reports in the literature have demonstrated the successful development of antibodies against eumelanin and pheomelanin metabolites.

If this antibody 6D4 can be successfully labeled with a radioactive isotope, it may be useful in other areas also; for example, it could be used for the development of radioimmunoscintigraphy of malignant melanoma (142) and as a carrier for conjugating cytotoxic agents in 'targeted' chemo-immunotherapy (143). Moreover, in basic science, locating the reactivity of MoAb 6D4 within various cell organelles fractionated from melanocytes could be used to study the biosynthesis of pheomelanin and its intermediates in normal epidermal melanocytes and diseased tissues (*e.g.*, melanoma cells).

Recent studies (144, 145, 146) have demonstrated that pheomelanin and its precursor, cysteinyldopa, are photochemically unstable when exposed to UVL; the photodegraded pheomelanin and cysteinyldopa can

generate free radicals, impair cell growth, and induce mutagenicity. MoAb 6D4 may be useful for investigating the production of UV-photoproducts of pheomelanin metabolites in tissues. Moreover, as 5-S-CD synthesis is enhanced in normal melanocytes after UV exposure and highly increased in transformed melanocytes (124), this new antibody can be used to estimate the risk of development of melanoma in patients who have familial DMN syndrome: a greatly increased 5-S-CD level after exposure to UVL would indicate a need for long-term follow-up.

On the other hand, this MoAb showed immunoreactivity with only three of eight histologic sections of malignant melanoma, all of the superficial spreading subtype. This unexpected finding may be due, at least partly, to the tissue fixation and dehydration processes. Alternatively, the antigenic epitope of 5-S-CD itself may be altered, or masked by conjugation with other metabolites (*e.g.*, GSH), in melanocytes and melanoma cells (56). These questions should be examined with immunostaining of frozen tissues.

In brief, a new MoAb 6D4 against a pheomelanin precursor, 5-S-CD, was developed—apparently the first MoAb against a melanin precursor. This antibody should provide a tool for studying the biology of melanin pigmentation and for developing new methods for diagnosing various pigmentary diseases, including malignant melanoma.

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